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(54) Title: HIGH THROUGHPUT CHROMATOGRAPHIC SYSTEMS
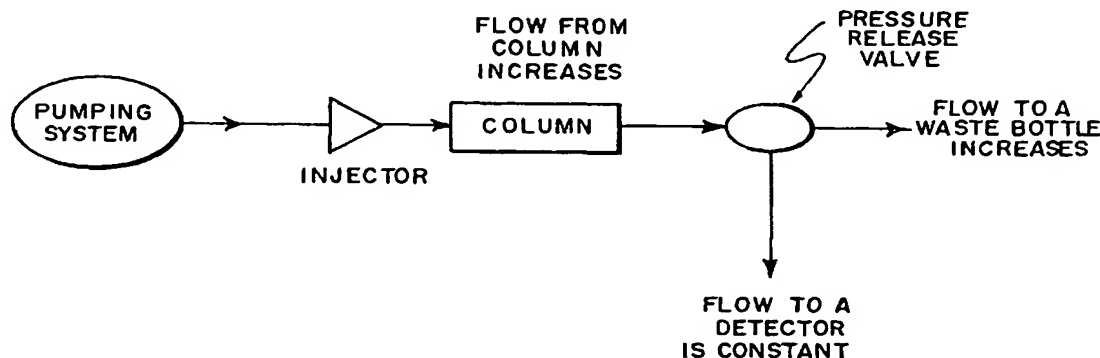
(57) Abstract: A method and apparatus is described for establishing a database comprising numerical values characteristic of the interaction of compounds and surfaces, such as stationary phases in a chromatographic system. In accordance with the invention a mobile phase flow gradient is used for enhancing efficiency and data precision in high throughput drug screening protocols. The chromatographic system includes an eluent flow splitter (10) in fluid communication with the chromatographic unit and the detector to minimize variation in flow rate to the detector with variation of rate of flow of the mobile phase is in the chromatographic unit. With use of a detector capable of providing a signal of the presence, or more preferably an identifying characteristic, of compounds eluting from the system, the invention provides a powerful method for creating a database of numerical values characteristic of the interaction of solute test compounds with a mobile phase and a stationary phase in at least two chromatographic separation systems. Data can be generated efficiently and stored in algorithm accessible electronic storage systems (databases) for use alone or in combination with other molecular descriptors for prediction of biological activity.

HIGH THROUGHPUT CHROMATOGRAPHIC SYSTEMS

Field of the Invention

This invention relates to the use of a chromatographic system for comparing physicochemical properties of compounds in a compound mixture. More particularly, the present invention is directed to a gradient flow chromatographic system for measuring and comparing numerical values characteristic of the interaction of compounds in a test mixture solution with a solid phase substrate. The gradient flow system offers advantages for acquiring and comparing data in connection with chemometric analysis and in high throughput drug screening protocols where the data is used alone or in combination with other molecular descriptors for prediction of biological activity.

Background and Summary of the Invention

The emergence of automated chemical synthesis platforms coupled with combinatorial techniques as a routine tool in the pharmaceutical industry has enabled the synthesis of large numbers of test compounds in a relatively short time. Millions of potential new drug candidates are produced every year, and both pharmaceutical and biotechnology industries have in recent years invested a significant effort to address the challenge of developing new, faster and more efficient ways to screen pharmaceutical compounds and to enable efficient identification and development of promising lead compound candidates. This, as well as the recent focus of the industry on related applications of chromatography to chemometric analysis, has created the need for high throughput analytical procedures for the collection of chromatographic data relevant to physicochemical and thermodynamic properties of compounds, and it has provided incentive for development of chromatographic systems specifically designed for automated high-throughput identification, purity assessment, purification and activity screening of combinatorial libraries and other compound mixtures including the analysis of biogenic, and more typically phytogenic, compound mixtures.

With the advent of combinatorial chemistry and the need to develop assays for the large numbers of compounds being made available using that

technology, many researchers have focused their efforts on developing in vitro tests/assays that provide biologically significant compound information. Much work has been directed to the correlation of certain physicochemical properties with biological activity, both in the search for new therapeutic agents and in the

5   understanding of compound toxicity from medicinal and environmental perspectives. For example, physicochemical properties of recognized significance to evaluation of a compound's biological activity are its lipophilicity, hydrophilicity, interfacial pKa, and membrane affinity among others. The determination of these properties is critical for QSAR studies, and the worldwide discovery effort. The present invention relates

10   to a method for efficient computer implemented determination of not only chemical structure, but also the physicochemical properties, derived from chromatographic data, critical for such QSAR studies and drug discovery efforts. More particularly, the present invention provides a method of choice for creating databases containing algorithm accessible data derived from chromatographic analysis of complex mixtures

15   needed for such QSAR studies.

Currently, automated semi-quantitative assessment of combinatorial libraries and other compound mixtures is most readily accomplished by coupling HPLC with UV detection and mass spectrometry. More recently product development efforts have been reported in the emerging area of microfluidics, wherein

20   submicrogram or nanogram quantities of compound mixtures are subjected to separation, analysis and even chemical modification in micron-dimensioned vessels and passageways etched in the surface of a solid substrate - the so-called "lab on a chip." While the present invention is described below particularly with reference to its application in HPLC systems, the concepts and principles of the invention will be

25   found to apply generally to the use of any chromatographic system used in drug discovery applications and chemometric analyses.

The use of chromatographic systems in such drug discovery methodology has limitations. Under isorheic chromatographic conditions, the compound components, both control compounds and test compounds, can have

30   widely distributed retention times (t_r), ranging from fractions of a minute to hours. While the retention times can be reduced by using mobile phase gradients of increasing or decreasing polarity (depending on the nature of the solid phase), changes

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in mobile phase composition can result in unpredictable variability in calculated k'
values. Such variability can be attributed to the differences in thermodynamic
equilibrium properties arising from varying the composition of the mobile phase.

Accordingly, researchers have focused their attention on optimizing
5   chromatographic throughput efficiency without resorting to use of mobile phase
gradient elution protocols and at the same time minimizing need for additional capital
expenditures. The work product of one such effort is described in PCT International
Publication No. WO 00/45929, published 10 August 2000. That publication describes
the development of a chromatographic system utilizing a pre-detector eluent switch
10   that allows multiple chromatographic columns to be operated in parallel using a single
eluent analyzer or detector. The eluent switch is a valve-containing device capable of
delivering small portions of the eluent from the individual chromatographic units to
the eluent analyzer, typically in a sequential or predetermined order. The eluent
switch permits the simultaneous determination of the HPLC profiles of a mixture of
15   compounds eluting from several chromatographic units operated in parallel. The
eluent switch allows cost-effective use of costly eluent analyzers (detectors), such as a
mass spectrograph, by incorporating a simple and relatively inexpensive switching
device immediately upstream (eluent flow-wise) from the eluent analyzer.

The separation of multi-component samples by LC under fixed
20   experimental conditions (i.e., normal elution) is often complicated by large differences
in the relative migration rates of the various components. This leads to poor
separation of the first eluted compounds and/or excessive separation times in addition
to difficult detection for the last eluted compounds. The need for shorter analysis time
and reduced peak width (resulting in a decrease in minimum detectable quantity)
25   prompted the development of gradient chromatography. At present, there are four
possible techniques of achieving gradient formation in liquid chromatography:
solvent programming, stationary phase programming (coupled columns), temperature
programming, and flow programming. Each of these various techniques is based on a
selective change in band migration rates during separation, such that strongly retained
30   bands are made to elute more rapidly than would be the case in normal elution.

Solvent programming (i.e., variation of the mobile phase composition
as a function of time) is widely used in chromatography as a way to speed up elution

time, mainly because it is so easy to achieve and control. However the scope of its

application is limited due to the fact that changing the composition of the mobile

phase affects a lot more than just its polarity/eluotropic strength. In particular, in case

where a buffer is used as part of the solvent system, changing the ratio buffer/organic

5   modifier affects the degree of ionization of the buffer species (i.e., the ionic strength

of the solution) and thus the pH of the solution. In addition, chromatographic surfaces

that contain ionizable functional groups will be sensitive to pH changes and/or ionic

strength of the mobile phase. Thus for this type of surfaces, solvent composition

gradients may change the local pKa at the interface between mobile phase and

10   stationary phase. Both the thermodynamics of the mobile phase/surface interactions,

and the chromatographic properties of the surface will be modified. Most

importantly, for ionizable solutes, the degree of ionization will change with mobile

phase composition, and so will their pKa. This has a significant impact on the scope

of applications for solvent programming techniques. In particular, this method cannot

15   be employed for the determination of physicochemical descriptors characteristic of the

solute/surface thermodynamic interactions or solute partitioning between stationary

and mobile phases, such as the capacity factor (k'). This observation has been

reported by Scott et al. (Scott R. P., Lawrence J. G., "The effect of Temperature and

Moderator Concentration on the Efficiency and Resolution of Liquid Chromatography

20   Columns", J. Chrom. Sci., 1969, 7, 65-71). A study of the dependence of retention

volume with organic modifier concentration for a group of 9 compounds was

conducted. It was shown that the dependence was not linear but parabolic, and that

the dependence was compound specific. The inventors confirmed that solvent

gradient elution is impractical, as a simple correlation between retention volume and

25   the solvent gradient profile cannot be established (Fig. 20).

Temperature programming is not often used in chromatography,

perhaps because the effects of temperature changes on separation are complex,

involving changes in both solute retention and band broadening. Nearly all the

physical parameters that play a role in LC separation are a function of temperature.

30   Changing temperature strongly affects mobile phase viscosity and the degree of

ionization of buffer solutions. It has been also shown that temperature variation may

induce stationary phase conformation transition, and the observation of this

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physicochemical phenomenon has initiated the development of temperature-responsive stationary phases as an additional tool for chromatographic selectivity optimization. Most of all, temperature variation affects the thermodynamics of solute/stationary phase interactions. Therefore, temperature programming is restricted

5   to applications where the purpose of the chromatographic run is not the collection of physicochemical parameters or other descriptors of the dynamics of the binding/interaction of the solutes with the chromatographic surface.

Flow rate programming as a means to speed up separation time in liquid chromatography is seldom used and has found limited applications in HPLC.

10   Studies involving flow gradient elution have been reported, but they remain scarce and limited to very specific cases. The potential of gradient flow elution in chromatography has been largely overlooked. Flow programming has not been used as a general method for accelerating data acquisition, minimizing instrument time, or for the collection of chromatographic databases (large numbers of chromatographic

15   parameters characteristic of physical, chemical or biological properties of the compounds studied). Several reasons to explain this trend in the chromatographic field are (1) organic modifiers are more efficient for eluting compounds, (2) the volume of mobile phase needed to elute compounds from the columns increases with flow gradients (more waste solvent to dispose of, and higher costs associated with

20   higher volumes of mobile phase), and (3) pressure changes to the column during flow gradients may reduce column lifetimes. However, the primary reason is that scientists did not (and still do not) believe it made chromatographic sense to decrease solute retention times using flow gradients.

There has been much research and development effort directed to the

25   definition of screening methods capable of identifying drug leads. The goal of such efforts is to define efficient methodologies for predicting biological activity *in vivo* by using empirically definable (or calculatable) descriptors to predict biological activity without the time investment and expense in costly animal studies. For many years most drug screening methods were based on conventional biological activity assays.

30   Generally drug leads are generated by comparing biological and physicochemical properties of test compounds with known compounds having recognized biological activity *in vivo*. There is a significant body of literature directed to prediction of

biological activities based on comparison of physical, chemical and biological descriptors and the use of pattern recognition analysis of such descriptors as part of drug screening protocols. In particular, membrane binding properties have been used as an assessment tool for biological activity screening of compounds. The method is based on the premise that compounds with similar sets of membrane binding properties will have similar pharmacological properties and/or biological activities.

The membrane binding properties of test compounds can be calculated, or they can be determined empirically with use of, for example, liposomes, immobilized artificial membranes, such as those described in U.S. Patent No. 4,931,498, Langmuir Blodget films, computer chips or similar devices with immobilized lipids, capillary zone electrophoresis columns coated with membrane lipids, and the like. In the case of immobilized artificial membranes (IAMs), the numerical values characteristic of membrane affinity are determined chromatographically using an aqueous mobile phase and a stationary phase comprising a membrane mimetic surface as defined in U.S. patent 4,931,498. Membrane binding properties of a set of test compounds of unknown biological activities are compared to the membrane binding properties of control compounds having known *in vivo* biological activity to assess the probability that the test compounds will exhibit one or more biological activities *in vivo*. For each control compound there is a defined and ordered set of numerical values characterizing a biologically relevant interaction (e.g., affinity) of that compound with each of the selected membrane mimetic surfaces.

The ordered set of numerical values for each control compound or each set of control compounds (i.e., a "training set") can be represented by the expression $<C_1, C_2, ..., C_n>$ wherein n is the number of membrane mimetic surfaces identified and used in the screening method. A similar ordered set of numerical values $<T_1, T_2, ..., T_n>$ for each test compound characteristic of its biological relevant interaction with each of the respective membrane mimetic surfaces is determined. The set of numerical values for each test compound is then compared with the set of respective values for the control compounds, and the biological properties of those control compounds having ordered sets of numerical values best matching the respective numerical values in the ordered set of values for the test compound are identified.

Pattern matching using vector calculus, multivariate analysis or principal component analysis of the numerical values characteristic of the test compounds and the control compounds allows comparison of the membrane binding properties of the test compounds and each of the control compounds or, if the control compounds all have a

5   common biological activity/property, average or mean membrane binding values of the set of control compounds for each membrane mimetic surface. Such drug discovery protocols are described in detail in PCT International Publication No. WO 99/10522.

An important criterium for optimum implementation of the use of

10   IAMs substrates for providing reliable values characteristic of membrane interaction include the use of identical mobile phase to elute both test and control compounds, as exemplified by the study conducted by Barbato et al. ("Chromatographic Indexes on Immobilized Artificial Membranes for Local Anesthetics: Relationships with Activity Data on Closed Sodium Channels", Barbato F., La Rotonda M. I., and Quaglia F.,

15   Pharm. Res., 1997, Vol. 14, No. 12, 1699-1705). As part of the study, the authors tried to establish a correlation between log k_w^{IAM} (w signifies 100% aqueous mobile phase) and log P for a set of 13 compounds. However, some compounds did not elute within a reasonable time with completely aqueous mobile phase. Hence, they had to be eluted with mobile phases containing various acetonitrile fractions (ϕ). A linear

20   relationship between log k'_{IAM} and ϕ was found (Fig. 1), and log k_w^{IAM} for these compounds was extrapolated to 0% acetonitrile.

Note that the linear plots of log k'_{IAM} versus ϕ have different slopes for each compound. In fact, when the lines intersect, as shown on Fig. 1, the order of elution changes. Table 1 lists the order of elution of each compound at the various

25   acetonitrile fractions. The elution time of a compound on the IAM surface is characteristic of its affinity for this membrane mimetic surface. An ordered set of numerical values reflecting the elution times of a particular compound on several IAM surfaces is termed membrane affinity fingerprint (MAF). In Table 1, the term "MAF X% ACN" is to denote that the elution order of the 13 compounds on the IAM surface

30   used for the study reflects their MAFs for this IAM surface. The highlighted compounds (bold characters) are the compounds for which a switch in elution order occurs. In this particular example, changes in elution order occur three times. This

implies that the elution time or capacity factor at different fractions of acetonitrile is not affected to the same extent for each compound. This has a significant effect on data acquisition for creating chromatographic databases, particularly when the data to be incorporated in the database are membrane affinity fingerprints (MAFs).

Table 1

Order	MAF 10% ACN	MAF 20% ACN	MAF 25% ACN	MAF 30% ACN
1	Procaine	Procaine	Procaine	Procaine
2	Gea 968	Gea 968	Gea 968	Gea 968
3	W 36017	Trocainide	Trocainide	Trocainide
4	Trocainide	W 36017	W 36017	W 36017
5	Prilocaine	Prilocaine	Prilocaine	Prilocaine
6	Lidocaine	Mepivacaine	Mepivacaine	Mepivacaine
7	Mepivacaine	Lidocaine	Lidocaine	Lidocaine
8	Trimecaine	Trimecaine	Trimecaine	Trimecaine
9	Bupivacaine	Alprenolol	Alprenolol	Alprenolol
10	Alprenolol	Bupivacaine	Bupivacaine	Bupivacaine
11	Etidocaine	Etidocaine	Etidocaine	Etidocaine
12	Tetracaine	Tetracaine	Tetracaine	Tetracaine
13	Propranolol	Propranolol	Propranolol	Propranolol

The MAF of a compound is defined as an ordered set of n numerical values (a vector) characteristic of the affinity of the compound for n membrane mimetic surfaces or Immobilized Artificial Membranes (IAMs). See WO 99/10522 for a detailed description. Figs. 2a-b illustrates how a change in elution order affects the classification/comparison of compounds in membrane space. Let us assume that, at a given mobile phase composition, compounds A and B are characterized by vectors [1, 3, 2] and [2, 1, 2] respectively (Graph 2a), in the three-dimensional membrane space (coordinate system) defined by IAM1, IAM2, and IAM3 (the numerical values defining the vectors reflect the elution time or affinity of the compounds for each surface: higher values mean higher retention/affinity). Suppose that compounds A and B were eluted on the same surfaces with a different mobile

phase composition, such that the elution of A and B on IAM 2 is reversed: A elutes before B (for simplicity considerations, let us assume that the retention of both compounds is not significantly changed on IAM 1 and IAM 3). The MAF vectors of compounds A and B are now [1, 2, 2] and [2, 3, 2], respectively. The change in
5   elution order has dramatically altered the position of each compound in membrane space, as shown in Figs. 2a-b. The process of membrane affinity fingerprinting (MAFing) is based on the comparison of MAF vectors in membrane space. As illustrated in Figs. 2a-b, a change in elution order causes compounds A and B to be defined by radically different vectors, which is unacceptable for MAFing purposes,
10   because it may lead to misclassification.

        The value of a chromatographic database is reflected not only by the nature/type of information that it contains, but also by the range of compound diversity that it encompasses. Collecting data for creating meaningful and robust chromatographic databases implies that compounds be eluted faster: (1) to minimize
15   data collection time and (2) to include strongly retained compounds. People routinely resort to solvent gradients to reduce compounds elution times. However, as extensively discussed above, this chromatographic technique implies non-linear dependence of retention volumes or capacity factors of the solutes with mobile phase composition, and a change in chromatographic behavior of the solutes, which is
20   compound specific.

        This is of particular significance for the collection of accurate data for chromatographic databases, particularly for these chromatographic descriptors characteristic of solute/surface thermodynamics. The major problem is that establishing a meaningful chromatographic database (with data that can be compared
25   from compound to compound) requires the use of a single mobile phase composition. This is not feasible because most compound libraries (organic, inorganic, from natural product sources, or combinatorial chemistry) comprise compounds with great structural and chemical diversity. Consequently, while a certain mobile phase composition may be adequate for the isorheic (constant flow) chromatographic elution
30   of a group of compounds on a given surface, it may not allow the elution of another set of compounds (for example higher retained solutes) in a reasonable amount of time. Under such conditions, a chromatographic database may be established only

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with compounds having similar affinities for the chromatographic surfaces used to build said database, excluding the many other compounds with significantly different affinities for the same surfaces.

A suitable solution to this problem would be to find chromatographic conditions that would allow the use of constant mobile phase composition (isocratic conditions) for all the compounds comprising the database, regardless of their relative affinities for the chromatographic surfaces to be used. The retention volume of any given compound is independent of the flow rate used to perfuse the column (for any given mobile phase composition, the volume of mobile phase required to elute a compound of a column is a constant, and does not depend on the flow rate). In a given compound mixture, compounds may elute from columns after very small or very large volumes of mobile phase have been perfused through the columns. In other words, compound mixtures may contain compounds with very low and very high affinity for a chromatographic surface. This makes it difficult or impossible to find one flow rate that will allow both early and late eluting peaks to be detected, and all the peak shapes to be characterized. For example, consider a compound mixture that contains 5 compounds that elute early (C1, C2, C3, C4, and C5) and 5 compounds that elute late (C6, C7, C8, C9, and C10) from a column with a 0.2 mL void volume. Furthermore, assume that the elution volumes and elution times at a flow rate of either 0.5 mL/min or 4.0 mL/min are:

Early eluting compounds

C1	C2	C3	C4	C5	Compounds			
0.2	0.4	0.6	0.8	1.0	Vr (mL)			
Acceptable peaks:				0.4	0.8	1.2	1.6	2.0	tr(min)0.5mL/min
Narrow unresolved peaks:				0.05	0.1	0.15	0.2	0.25	tr(min) 4mL/min

Late eluting compounds

C6	C7	C8	C94	C10	Compounds				
100	120	140	160	180	Vr (mL)				
Broad peaks, poor detection:					200	240	280	320	360	tr(min)0.5mL/min
Acceptable peak shape:					25	30	35	40	45	tr(min) 4mL/min

As exemplified above, at 0.5 mL/min the early eluting compounds will easily be detected and characterized, whereas the late eluting peaks will likely be so broad that they will be difficult to detect by any method. Furthermore, at 4.0 mL/min the early eluting peaks elute so quickly that the peak shapes can not be characterized and that retention times are difficult to measure, but the late eluting compounds are easily characterized. Thus getting a complete set of chromatographic data for the compound mixture C1-C10 would require two chromatographic runs: one at 0.5 mL/min and one at 4.0 mL/min.

A better approach yet is to collect the data with a flow gradient, ramped or stepped from 0.5 mL/min to 4.0 mL/min. Chromatographic data for both early and late eluting compounds could be obtained with acceptable peak shape characterization and peak detection, all of which being accomplished in one single run and in a minimum amount of time.

The present invention focuses on the use of flow gradients for establishing chromatographic databases (e.g., the collection of large numbers of chromatographic data characteristic of chemical, physical, biological and/or thermodynamic properties of solutes) for mixtures of chemically diverse compounds exhibiting a broad range of affinities for the chromatographic surface in use. The primary advantage of the present invention is that it provides a solution to a problem routinely encountered in the field of chromatography: the non-linear dependence of solute retention time with mobile phase composition (in particular organic modifier concentration). This phenomenon, due in part to a change in the thermodynamics of solute/stationary phase interactions, makes the use of solvent gradients (modification of the mobile phase composition as a function of time) or organic modifier extrapolation techniques unsuitable for the characterization of said thermodynamic interactions for mixtures of compounds with radically different chromatographic

affinities (i.e., poorly retained compounds versus solutes with high retention). This problem has high significance for disciplines such as chemometrics, determination of physicochemical properties, prediction of biological properties, and drug discovery in general. The flow gradient method described herein allows the efficient, reliable, and

5   accurate determination of chromatographic parameters (characteristic of solute thermodynamic properties) for a wide variety of structurally, physicochemically, and biologically diverse compounds. Thus, it is particularly suitable for the collection of large databases containing empirical data derived from chromatographic analyses.

In addition, the present invention provides a chromatographic method

10   that allows faster elution of higher retained solutes. In one embodiment of the invention, a chromatographic system allowing highly efficient determination of physicochemical properties and/or chemical structure identification, through the use of a chromatographic unit (capable of delivering an accurate flow gradient according to a pre-set profile) in communication with one eluent analyzer, is described. Such a

15   system dramatically increases the performance, efficiency, scope of use and commercial value of said chromatographic unit/eluent analyzer system. The chromatographic unit can be any chromatographic system that can be interfaced with an analyzer or detector, and can include (but is not limited to) high-performance liquid chromatography (HPLC) columns, capillary electrophoresis chromatography (CEC)

20   columns, Gas Chromatography (GC) columns, super-critical fluid columns and microchips. The eluent analyzer unit is any instrument capable of identifying the presence, physicochemical characteristics and/or chemical structure of a compound, including (but not limited to) a mass spectrometer (MS), a Fourier transform infra red spectrometer (FTIR), a Fourier transform ultra violet spectrometer (FTUV), standard

25   UV detector, fluorescent detector, electrochemical detector, and a Fourier transform nuclear magnetic resonance spectrometer (FTNMR). The present design permits faster collection of the HPLC profiles (including structural identification) of a mixture of multiple compounds eluting from one chromatographic unit. The implementation of the method described therein allows a more efficient and cost-effective use of a

30   costly eluent analyzer unit (such as, MS).

The chromatographic process represents a reversible equilibrium of solutes between a mobile phase and the stationary phase in a chromatographic system.

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The magnitude of the solute retention is a direct result of this equilibrium, and it is typically expressed by a parameter known as the capacity factor, k'. In an isorheic (constant flow) system k' is equal to $(t_R-t_0)/t_0$ where t_0 is the dead time of the chromatographic unit and t_R is the retention time of the respective solutes.

5   Alternatively the capacity factor can be expressed as a function of V_0, the so-called dead volume of the chromatographic unit, and V_R the retention volume, i.e., the volume of mobile phase required to elute the respective solute from the chromatographic unit, e.g., an HPLC column. Thus the capacity factor can be calculated as $k'=(V_R-V_0)/V_0$. The capacity factor is therefore a numerical value

10   characteristic of a mass distribution equilibrium of the solutes between the mobile phase and the stationary phase in a chromatographic unit, and its determination allows the calculation of various physicochemical values according to pre-determined algorithms. It has been reported that comparison of k' values of test compounds of known biological activity in chromatographic units, e.g., those utilizing multiple

15   unique solid phase substrates, can be compared with the k' values (most preferably with vectors representative thereof) for compounds of known biological activity. While art-recognized standard HPLC solid phase substrates can be employed, this chromatographic technique for predicting the properties of test drug substances has been found to be particularly effective when the solid phase substrates are selected to

20   have membrane mimetic properties. The use of k' values calculated for test compounds and control compounds based on use of multiple chromatographic units using unique stationary phase substrates to predict biological properties is detailed in PCT Publication No. WO 99/10522 published 04 March 1999.

In programmed flow rate operation, a linear program rate is often used,

25   but the method is not limited to this flow rate profile. The various flow rate programs that may be used in chromatography can be classified as ramp, step and multi-segment. The initial flow rate, the final flow rate, and the programming rate define the theoretical program (equation). The retention volume of a specific solute is a constant value at any flow rate. Knowledge of the mathematical expression

30   describing the variation of flow rate as a function of time allows the determination of the retention volume of any compound at any given time. The retention volume of a

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specific solute under gradient flow conditions can be calculated by integrating the time profile of the employed flow rate programming pattern.

The present invention derives from a continuing effort to enhance throughput in chromatography-based drug discovery/chemometric methodologies. It is based on use of a gradient flow elution protocol for the expedient determination of k' values and other numerical values characteristic of the interaction of test compounds and control compounds with a solid phase substrate. The present chromatographic system and method eliminates the need to vary mobile phase component ratios and the consequent interference with the determination of numerical values characteristic of the interaction of the test and control compounds (i.e., the solutes and the mobile phase), with the surface of the stationary phase. The improved performance of the present system derives from use of variable elution flow rate chromatographic system capable of measuring and recording the retention volume (V_R) for each solute eluted from the chromatographic unit.

Thus there is provided in accordance with the present invention a chromatographic system for comparing and or determining physicochemical properties of compounds in a sample including a mixture of compounds, particularly represented as numerical valves characteristic of the interaction of the compounds with the stationary phases in the chromatographic system. The mixture can include test compounds and one or more compounds of known biological activity. The chromatographic system comprises a chromatographic unit having a sample loading port, a mobile phase delivery port, an eluent exit port, and a stationary phase. The stationary phase can be any commercially available stationary phase detailed for use in chromatographic applications. Preferred stationary phases include the membrane mimetic surfaces described in U.S. Patent No. 4,931,498, and, for example, immobilized macromolecules.

The improved chromatographic system of the present invention further comprises a mobile phase supply system including a pump (mechanical, osmotic, etc.) and a source of mobile phase for delivering the mobile phase to the mobile phase delivery port of the chromatographic unit. In one embodiment of the present invention the chromatographic unit is in the form of a column containing a solid phase of the sort designed for use in classical HPLC systems.

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The present chromatographic system also includes a detector in fluid flow communication with the eluent exit port. The detector has an eluent sampling port and is capable of providing a signal of the presence or identity of a compound in eluent delivered to the detector sampling port. Preferably the detector is capable of

5   providing a signal characteristic of the detected solute compound. Thus the detector can comprise a mass spectrometer, a Fourier transform infra-red spectrometer, a Fourier transform ultraviolet spectrometer, or a Fourier transform nuclear magnetic resonance spectrometer. In one preferred embodiment the detector comprises a mass spectrometer (MS). The MS detector provides a signal of total ion concentration in

10   aliquots of eluent delivered to the sampling port of the detector.

Another element of the present chromatographic system is a controller for the pump component of the mobile phase supply system for controlling the flow rate of mobile phase through the chromatographic unit at a programmed flow rate. The controller is also designed to provide signals indicative of the volume of mobile

15   phase delivered to the chromatographic unit after delivery of the sample to the unit. Alternatively, and less preferably, the chromatographic system includes a flow rate reporter and a timer for providing signals indicating the time following delivery of a sample to the sample delivery port. Signals from the timer and the flow rate reporter can be directed to a microprocessor programmed to calculate the volume of mobile

20   phase delivered to the chromatographic unit at any point in time. The controller for the pump is programmed to modulate the rate of flow of mobile phase through the chromatographic unit according to a predetermined flow rate profile. The profile can, for example, be ramped and represented by the formula flow rate = a + bt wherein "a" is the initial flow rate, "t" is the time after sample delivery, and "b" is the slope of the

25   plot of flow rate versus time. Alternatively the controller can be programmed to adjust the flow rate in a stepped profile.

The chromatographic system of the present invention further comprises a data management system in communication with the pump controller and the detector. The data management system comprises a data storage unit for storing

30   signals from the detector as a function of volume of mobile phase delivered to the chromatographic unit. Optionally it can include a programmable microprocessor. In one embodiment the data management system is programmed to include an algorithm

and mass spectral data for identifying the signals from the detector for the individual

detected compounds to convert total ion concentration values reported by the detector

to what is termed an extraction ion chromatogram as a function of retention volume.

The programmable controller and data management systems for use in the present

5   invention are generally those available on commercially available HPLC systems, for

example, or such elements can be easily modified or reprogrammed to meet the

processing requirements of the various applications of the present invention.

The chromatographic system of this invention can also include an

eluent flow splitter in fluid communication with the eluent exit port and the detector

10   sampling port to minimize variation in flow rate to the detector as a function of the

rate of flow of mobile phase through the chromatographic unit. The eluent flow

splitter includes a chamber having an eluent inlet and two eluent outlets, one in fluid

flow communication with a waste collection vessel through a pressure relief valve or

related device and the other in fluid flow communication with the sampling port of the

15   detector. The eluent flow splitter finds particular application when the detector

comprises a mass spectrometer. Such detectors often require regulated rate of flow

delivered to the sample port for optimum detection efficiency. The flow rate of eluent

from flow splitter to the detector is a function of the setting of the pressure relief valve

on the splitter and the internal diameter of the conduit or orifice communicating with

20   the detector sampling port.

In another embodiment of the invention the chromatographic system

includes a display device in communication with the data management system for

reporting the detector signal as a function of retention volume. The data management

system can also include a microprocessor programmed to calculate and store k' values

25   for at least a portion of the eluted compounds wherein $k' = (V_R-V_o)/V_o$ wherein V_R is

the retention volume for an eluted compound and V_o is the dead volume of the

chromatographic unit. The data management system can also be coupled to a data

output device for reporting the k' value for at least a portion of the compounds eluted

from the chromatographic unit. In addition, the data management system can include

30   a processor programmed with an algorithm for calculating other chromatographic

parameters and/or descriptors (in addition to k' values) such as peak shape, peak

asymmetry, median, mean, peak skewness, etc. Alternatively, a program may be

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incorporated that converts point by point a chromatogram experimentally obtained under a given flow gradient profile (constant, stepped, ramped or multi-segment) into a putative chromatogram that would be obtained under other (theoretical) flow gradient conditions.

In one embodiment of the invention the chromatographic system includes at least two chromatographic units, each having an effluent flow splitter communicating with the eluent exit port and a programmable eluent switch located between the outlets (to the detector sampling ports) of each eluent flow splitter and the sampling port of the detector. The eluent switch is capable of receiving eluent from each of the splitters and delivering aliquots of eluent from each of the eluent flow splitters to the sampling port of the detector, said delivered aliquots optionally separated by an aliquot of a reference fluid from a source thereof, also in fluid flow communication with the eluent switch.

Use of the improved chromatographic system of this invention enables enhanced performance of chromatography-based drug delivery and/or chemometric analysis protocols. In one preferred use of the present chromatographic system test compounds can be screened for biological properties by combining them with a training set composition comprising one or more control compound having a common biological property to provide a test mixture. A portion of the test mixture is subjected to chromatographic separation in a chromatographic system comprising a stationary phase and a mobile phase to provide numerical values characteristic of the interaction of the compounds in the test mixture with the stationary phase. The numerical values of the test compounds are compared with those of the control compounds to identify the test compounds having values that best match those of the control compounds. Use of the non-isorheic chromatographic system of this invention enables highly efficient data acquisition.

Compound purification can be accomplished using the present invention. For example, multiple test compounds are eluted from two or more chromatographic units and aliquots of the eluent are delivered to the detector via the eluent switch, which is configured so that the "waste" (i.e., that portion not delivered to the detector) is collected in a fraction collector from each respective chromatographic unit. The fraction collectors are coordinated with the time of

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detection such that the appropriate fraction containing any detected compound can be identified. When a compound with predetermined physicochemical properties is identified, the appropriate fractions containing a purified form of the identified compound are retained from the fraction collector(s).

5   The invention also finds use in the analysis, identification and purification of compounds of widely variant activity, i.e., activities ranging from pharmaceutical utility to toxicity. For example, determination of physicochemical characteristics of biologically active compounds, biologically toxic compounds and environmental toxins such as pesticides, herbicides, etc., can be accomplished using
10   the present invention.

         In another embodiment, compound analysis is performed on the eluted sample compounds to determine their chemical structure. For example, compounds with predetermined physicochemical properties can be identified using the present invention. The present invention provides an embodiment wherein a library of
15   structurally related compounds is eluted from two or more chromatographic units. Aliquots of the eluent from each column are delivered to a detector capable of determining both the presence and the structural identity of the eluted compounds. The signals generated by this detector are evaluated, and elution profiles and/or other physicochemical properties of the eluted compounds are determined. In this manner,
20   compounds with elution profiles matching the predetermined physicochemical properties desired are selected and identified.

         The chromatographic system can include two or more chromatographic units operated in parallel using a common or independent pumps and programmable pump controllers. The numerical values can be collected and stored electronically in
25   algorithm-accessible memory for display or use in calculating related values and for performing calculations for comparing the numerical values for the individual test compounds with those in the training set or sets of compounds used to form the respective test mixtures.

         The present invention thus provides a method for creating a database
30   for numerical values characteristic of the interaction of solute test compounds with a mobile phase and a stationary phase in a chromatographic system, most typically in at least two chromatographic separation systems. The method comprises the step of

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selecting a mobile phase, a stationary phase, and an initial mobile phase flow rate independently for each chromatographic separation system. One or more compounds are delivered to the chromatographic separation systems for separation. Significantly, the flow rate of the mobile phase is changed in at least one of the chromatographic

5   separation systems during separation of the compounds. At least a subset of the compounds are detected as they elute from the column and signals indicative of compound elution, more specifically numerical values indicative of the elution volume of the eluting compound, the elution peak profile, or a value derived from said values for each detected compound is stored in a predefined data array in an electronic

10   database. The database is typically accessible by an algorithm capable of reading said data and processing it to provide information comparing the retention volume and other values characteristic of the compound's interaction with the stationary phase with those values for compounds having known biological activity.

Preferably the detector on the chromatographic separation system is a

15   spectrophotometer and the eluted compounds are subjected to spectral analysis as they are eluted from the system. Numerical values derived from said spectral analysis of the eluted compounds are stored as a function of the retention volume for the eluted compounds in the electronic storage device. The numeric values characteristic of the interaction of the eluted solute compounds between the mobile phase and the

20   stationary phase in the chromatographic system typically comprises the respective retention volume of the eluted compounds or values derived from algorithmic   —
manipulation of data relating to elution peak profiles, such values including capacity factor, peak width, standard deviations, peak skewness, peak asymmetry, peak kurtosis, and other properties that can be calculated from chromatographic peak data.

25   The method can also be conducted to produce numerical values for specific compound/stationary phase interactions, for example, by conducting chromatographic separations using mobile phases of unique pH, running multiple separations wherein the chromatographic systems are each held at unique temperatures, or running multiple separations wherein the mobile phases are of unique

30   ionic strength. Typically the flow rate is increased during data acquisition to obtain numerical values for multiple compounds from a single injection. Fractions of the eluting compounds can be collected as part of the chromatographic separation process

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and thereafter the various eluting compounds can be analyzed independently for biological activity.

Brief Description of the Drawings

5   Fig. 1 is a plot of logarithm of capacity factors of a group of 13 compounds determined on an IAM column at different fractions of acetonitrile. (Barbato, F. et al., Pharm. Res., 1997, Vol. 14, No. 12, 1677-1705).

Figs. 2a-b illustrate the consequences of changing compound elution order in membrane affinity fingerprinting (MAFing).

10   Fig. 3 is a diagrammatic representation of the relationship of the elements of the chromatographic system in accordance with one embodiment of the invention.

Fig. 4a illustrates mass chromatograms (total ion chromatogram (TIC) and Fig. 4b illustrates extracted ion chromatogram (EIC) of a mixture of drugs under

15   isocratic flow conditions. Mass chromatograms (total ion chromatogram (TIC) and extracted ion chromatogram (EIC)) of a mixture of 15 drugs under isocratic flow conditions that are described in the experimental section. 1, atenolol; 2, minoxidil; 3, indoprofen; 4, acebutolol; 5, nalbuphine; 6, minaprine; 7, 8-hydroxy-2-(di-n-propylamino)tetralin (8-OH-DPAT); 8, estazolam; 9, buspirone; 10, flunitrazepam;

20   11, cyheptamide;12, chlordiazepoxide; 13, trazodone; 14, diazepam; 15, delorazepam.

Figs. 5a-b are similar to Figs. 4a-b showing chromatograms of the same drug mixture under gradient flow conditions. Mass chromatograms (total ion chromatogram (TIC) and extracted ion chromatogram (EIC)) of a mixture of 15 drugs under gradient flow conditions that are described in the experimental section. 1,

25   atenolol; 2, minoxidil; 3, indoprofen; 4, acebutolol; 5, nalbuphine; 6, minaprine; 7, 8-hydroxy-2-(di- n-propylamino)tetralin (8-OH-DPAT); 8, estazolam; 9, buspirone; 10, flunitrazepam; 11, cyheptamide; 12, chlordiazepoxide; 13, trazodone; 14, diazepam; 15, delorazepam.

Figs. 6a-b show a comparison of capacity factors (k') from isocratic

30   flow and gradient flow protocols. Fig. 6a shows the data in tabular form, while Fig. 6b shows a plot of the data.

Figs. 7a-b show a comparison of peak starting points from isocratic flow and gradient flow profiles. Gradient flow peak starting times are normalized/converted to 1 ml/min flow rate after calculation of the corresponding retention volume. Fig. 7a shows the data in tabular form, while Fig.7b shows a plot of

5   the data.

Figs. 8a-b show a comparison of peak ending points from isocratic flow and gradient flow profiles. Gradient flow peak ending times are normalized/converted to 1 ml/min flow rate after calculation of the corresponding retention volume. Fig. 8a shows the data in tabular form, while Fig. 8b shows a plot

10   of the data.

Figs. 9a-b show a comparison of peak width from isocratic flow and gradient flow profiles. The base peak widths (i.e., peak width at -4.4% peak height, e.g. 5 x σ_{SD}) for both isocratic and gradient flow are listed. The base peak width ($W_{4.4}$) is defined as the intersection of the tangents at the inflection points of the peak

15   of interest with the baseline. Fig. 9a shows the data in tabular form, while Fig. 9b shows a plot of the data.

Figs. 10a-b show a graphic comparison of capacity factor (k') from isocratic and gradient acetonitrile elution profiles. Fig. 10a shows the data in tabular form, while Fig. 10b shows a plot of the data.

20   Figs. 11a-b show a comparison of peak starting points from isocratic and gradient acetonitrile elution profiles. Fig. 11a shows the data in tabular form, while Fig. 11b shows a plot of the data.

Figs. 12a-b show a comparison of peak ending points from isocratic and gradient acetonitrile elution profiles. Fig. 12a shows the data in tabular form,

25   while Fig. 12b shows a plot of the data.

Figs. 13a-b show a graphic comparison of peak width from isocratic and gradient acetonitrile elution profiles. The base peak width ($W_{4.4}$) is the peak width determined at 4.4% peak height and is -5 x σ_{SD}. Fig. 13a shows the data in tabular form, while Fig. 13b shows a plot of the data.

30   Fig. 14 illustrates the construction of a flow splitter utilized in one embodiment of the present invention.

Fig. 15 is a diagrammatic representation of a chromatographic system of this invention wherein 3 columns are used to generate compound data for electronic storage and later use in drug discovery protocols.

Figs. 16a-d comprise a graphic comparison of the capacity factors (k') of 10 compounds between 0% acetonitrile (ACN) isocratic conditions versus 5% (Fig. 16a), 10% (Fig. 16b), 20% (Fig. 16c), and 30% (Fig. 16d) ACN isocratic conditions respectively.

Figs. 17a-c are a graphic comparison of the capacity factors (k') of the 10 compounds used for Figs. 16a-d between flow gradient and isorheic (constant flow) conditions at 10% (Fig. 17a), 20% (Fig. 17b), and 30% (Fig. 17c) acetonitrile.

Figs. 18a-c are a graphic comparison of the capacity factors (k') of the 10 compounds used for Figs. 16a-d between (Fig. 18a) two isorheic runs: 2 mL/min versus 1 mL/min, (Fig. 18b) two isorheic runs: 4 mL/min. versus 1 mL/min., and (Fig. 18c) a flow gradient and an isorheic run (1 mL/min.).

Fig. 19 is a graphic comparison of the capacity factors (k') of the 10 compounds used for Figs. 16a-d eluted with the same mobile phase composition (10% acetonitrile) under various flow rate conditions.

Fig. 20 shows capacity factors for nine compounds plotted against acetonitrile concentration.

Fig. 21 is similar to Fig. 20 except using varying salt concentrations.

Fig. 22 is a plot of the capacity factors (k') of 9 compounds (obtained by flow gradient elution: ramped gradient from 0.5 mL/min. to 4.0 mL/min over 30 min.) as a function of acetonitrile fraction in the mobile phase.

Fig. 23 is a graphic representation of the effect of salt concentration of the mobile phase in terms of k' values for seven compounds.

Detailed Description of Selected Embodiments of the Invention

As shown in Fig. 3, one embodiment of the invention is based on a chromatographic system containing a pumping system, at least one injector, at least one chromatographic column, at least one pre-detector pressure relief valve (depending on the type of detector used), and a detector which must be such that it allows identification and quantification of detected compounds alone or in mixtures

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with other compounds on a millisecond time scale. An HPLC is the preferred chromatographic support for this invention, although other chromatographic techniques are suitable for the purpose (e.g., CEC, microchips, GC). The preferred detection device is a mass spectrometer, although other detection systems, such as

5   FTIR, FTUV and FTNMR detectors are acceptable. The use of a tandem mass spectrometer (MS/MS) may be required for more complex cases where the identification of the compounds eluting from the columns is not possible after a single mass spectrometric analysis.

10   Proof of concept

     The flow gradient concept was initially tested with a set of 24 compounds, which were eluted (1) under isorheic conditions (constant flow) and (2) with a gradient flow, all other chromatographic parameters being the same. For constant flow experiments, the retention time was determined for each compound and

15   the corresponding capacity factors were calculated. For gradient flow experiments, each compound retention volume was determined, and the corresponding capacity factors were calculated. For each chromatographic peak, a start point and an end point were determined and were used to calculate the peak width ($W_{4.4}$) at 4.4% peak height (i.e., 5 σ). The peak start and end points were defined as the intersections of

20   the tangents at the inflection points of a particular peak with the chromatogram baseline. The capacity factors (k'), the peak start points, the peak end points and the peak widths ($W_{4.4}$) were determined for each compound for the isorheic run and for the flow gradient run. Correlation plots between constant flow and gradient flow data were established and confirmed that the chromatographic data obtained from the

25   gradient flow experiment matched nearly perfectly those obtained under constant flow conditions (see Figs. 6a-b through 9a-b).

     The acetonitrile gradient data (Figs. 10a-b through 13a-b) were conducted merely to illustrate the fact that solvent gradient protocols are not suitable when the experimental objective is to compare thermodynamic properties. Solvent

30   gradient techniques are preferred if the experimental purpose is quantitation, isolation, fractionation, or compound identification.

General Chromatographic Conditions

A chromatographic system similar to that described in Fig. 3 was used for the study. The detector was either a UV spectrometer or a mass spectrometer. Two HPLC ^{Ester}IAM.PC$^{C10/C3}$ columns (30 × 4.6 mm) were used. See WO 99/10522 for a detailed description of the indicated stationary phases. For the flow gradient and isorheic (constant flow) experiments, the mobile phase was 15% acetonitrile in 30 mM aqueous ammonium acetate (pH 7.4). The sample concentration was 1 µg/µL and the amount of sample injected was 10 to 20 µL. The column pressure was up to 170 bar (or about 2400 psi) when the flow rate reached nearly 4 mL/min.

Flow Gradient Setup

The choice of flow gradient profile depends on the type of analyte under consideration and its chromatographic behavior under a given set of chromatographic conditions. For example, the gradient can be ramped, stepped or multi-segment, and the gradient slopes can be steep or gentle. For instance, steep gradient flows can be used for mixtures of compounds that separate well, and gentle gradient flows can be used for those mixtures with analytes that partially co-elute or have similar retention times under a given isorheic protocol.

For our study, the 24 test compounds were grouped essentially in two sets: compounds with low affinity for the IAM surface (17 compounds total) versus compounds having higher affinity for the surface (7 compounds). Two variations of the same ramped gradient were used to elute each group of compounds. In one flow rate profile, the gradient was ramped from 0.5 ml/min. to 4 ml/min. over 30 min. This flow gradient method was used for both UV (2 compounds: Clozapine (16) and Imipramine (17)) and MS detection (compounds (1) to (15)). The second gradient flow method was identical to the first one except that after 30 min, a flow of 4 ml/min was maintained for an additional 3 hours. This flow gradient method was used for the 7 most strongly retained compounds (compounds (18) to (24), MS detection).

Acetonitrile Gradient

Compounds (1) to (17) were used to conduct the solvent gradient study. The compounds were eluted with a ramped acetonitrile gradient (from 5% to

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30% acetonitrile in 30 mM aqueous ammonium acetate over 30 minutes) at 1 mL/min. (the pH was not monitored during the run). The chromatographic parameters (k', peak start and end points, peak widths ($W_{4.4}$); Figs. 10a, 11a, 12a, and 13a, respectively) from the acetonitrile gradient experiment were plotted against the
5   corresponding parameters obtained with a 15% acetonitrile isorheic run (Figs. 6a-b (k'), 7a-b (peak start), 8a-b (peak end), and 9a-b ($W_{4.4}$), data displayed in the column labeled "isorheic flow"). As expected, very poor correlation can be found between k' values and peak widths between isocratic and gradient acetonitrile runs. The peak ending point data set seems to show some correlation. This is probably an artifact (the
10   rest of the chromatographic parameters shows no correlation).

Controlling the Flow Rate into the MS Detector System

         The MS system cannot tolerate high chromatographic flow because of both pumping capacity and detector sensitivity limit. The advertised maximum LC
15   flow rate tolerated by the Esquire-LC (the model of LC/MS instrument used for the validation studies supporting this patent) is 1.0 ml/min. Based on this consideration, when high LC flows (> 1.0 ml/min) are required for a particular study, the LC effluent needs to be split prior to entering the MS detector; thus, a piece of hardware (splitter 10), diagramed in Fig. 14 is installed immediately upstream of the MS inlet, keeping
20   the flow to the MS detector within the range tolerated by the instrument. The excess flow is sent to a waste bottle. When higher flow rates are used (for example, 4 ml/min.) most of the flow is directed to the waste bottle, and only a small portion of the flow (set by the user, i.e., 0.5 ml/min) is directed into the MS system. A flow splitter 10 (or controller) (Upchurch Scientific, Oak Harbor, WA) (Fig. 14) was used
25   to split the eluent flow and control the flow to the detector. The splitter 10 is composed of a back pressure regulator 16 (Upchurch part number P.790; 100 psi cartridge) assembled with a tee 14 (Upchurch part number P.612), and essentially functions as a pressure relief valve: the back pressure regulator 16 contains a cartridge which regulates the pressure of the incoming flow by preventing it from
30   exceeding a given pressure (set by the manufacturer or the user). Pressure relief cartridges are available from Upchurch with the following settings: 5, 20, 40, 75, 100, 250, 500 and 1,000 psi. If more flexibility is required, the cartridges may be modified

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to allow the operator to manually adjust the pressure setting to a desired value.
Although this kind of valve was initially designed for applications where back
pressure fluctuations are a problem (for example in systems that have delicate
components requiring a low-pressure threshold), the device may be applied to regulate
5   the flow to the MS inlet in an LC/MS system operating under gradient flow
conditions. Thus, the Upchurch splitter 10 was connected to different ID tubings.
The splitter outlet 22 sending flow to the MS system is connected to a small ID
tubing. When the flow through the splitter 10 increases, the pressure builds up until it
reaches the threshold. The excess pressure is relieved by an inline check valve 12 or
10   pressure release valve that is linked to a larger ID tubing. At low flow rates (i.e., 0.5
ml/min.) the pressure in the valve 12 does not exceed the preset limit, and most of the
eluent flows directly from the valve inlet 20 to the outlet 22 (through a small ID
tubing) in the MS system without activating the inline check valve 12. At high flow
rates (i.e., 4 ml/min.), the pressure is relieved by the inline check valve 12 and most of
15   the eluent is sent via waste outlet 24 to the waste bottle through the larger tubing, and
only portion of the eluent goes to the MS system via the small ID tubing.

         The flow to the MS system was tested at two different flow rates, 0.5
and 4.0 ml/min. At 0.5 ml/min, the flow to the MS system is 0.4 ml/min. At 4.0
ml/min., the flow to the MS system is 0.8 ml/min.
20

General Mass Spectrometer (MS) Conditions

         An Esquire-LC model from Hewlett Packard and Bruker was used.
This LC/MS instrument is equipped with an orthogonal electrospray ionization (ESI)
source and an ion trap mass analyzer.
25   The source voltage was set at 3 kV; cap exit, 90 V; skimmer 1, 25V.
Low mass trap cutoff was set at 60 m/z, full scan range was set from 100 to 700 m/z.
Nitrogen drying gas was delivered at 10 ml/min. and the nebulizer pressure was set at
30.0 psi. The polarity and mode used in these experiments are positive and
standard/normal, respectively. The summation was 30 spectra.
30

Compounds Tested

Figs. 6a-b through 9a-b detail the test compounds used for the experiment, the chromatographic data collected under both constant and gradient flow conditions, and the associated correlation plots. The concept was initially tested with

5   Clozapine (16) and Imipramine (17) using a UV spectrometer as the detector. Additional data was collected with 22 other compounds (compounds (1) to (15) and (18) to (24)) using a mass spectrometer as the detection system.

Mathematical Treatment of the Data

10   The capacity factor (k') can be expressed as a function of retention time (t_r) or retention volume (V_r). Knowledge of the flow gradient profile (mathematical equation expressing the flow rate as a function of time) allows the calculation of the volume that has eluted through the column at any given time. Thus, the dead volume V_0 of a column and the retention volume V_r of each compound eluting from the

15   chromatographic system are easily accessible through a mere integration operation. The determination of V_0 and V_r allows the calculation of the capacity factors (k') for each compound (Equation 1).

$$k' = \frac{V_r - V_o}{V_o} \qquad \text{Eq. 1}$$

20

Under isorheic (constant flow rate) conditions, V_o can be calculated by multiplying the dead retention time (t_0) by the flow rate (f) (i.e., 1ml /min), and Vr is the analyte retention time (t_r) times the flow rate (f). [*Note that Eq. 1 can be simplified to Eq. 2,*

25   *which is commonly used by chromatographers*].

$$k' = \frac{t_r \times f - t_o \times f}{t_o \times f} \qquad Þ \qquad k' = \frac{t_r - t_o}{t_o} \qquad \text{Eq. 2}$$

30

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The retention volumes (V_r) and dead volume V_0 for the flow gradient experiments were calculated by integrating the mathematical function f(t) (expressing the flow rate f as a function of time) between t=0 (start of the run) and t_r (elution time of the analyte), and between t=0 and t_0, respectively (Equation 3).

5

$$V(t_1, t_2) = \int_{t_1}^{t_2} f(t)\,dt.$$ Eq. 3

Various flow gradient profiles are possible, and graphic representations of three examples are given below. Graph (a) represents the profile of a pumping
10   system giving out a constant flow rate (*f(t)* = *constant*). Graph (b) illustrates a ramped flow gradient profile, as used in the present study (i.e., *f(t)=a + bt*). Although not represented below, a step gradient or any combination of the above mentioned gradient types (multi-segment gradient) is also possible. Graph (c) is to illustrate that any flow gradient profile may be used, as long as its mathematical expression is
15   known and that the pumping system can deliver such gradient.

(a)   (b)   (c)
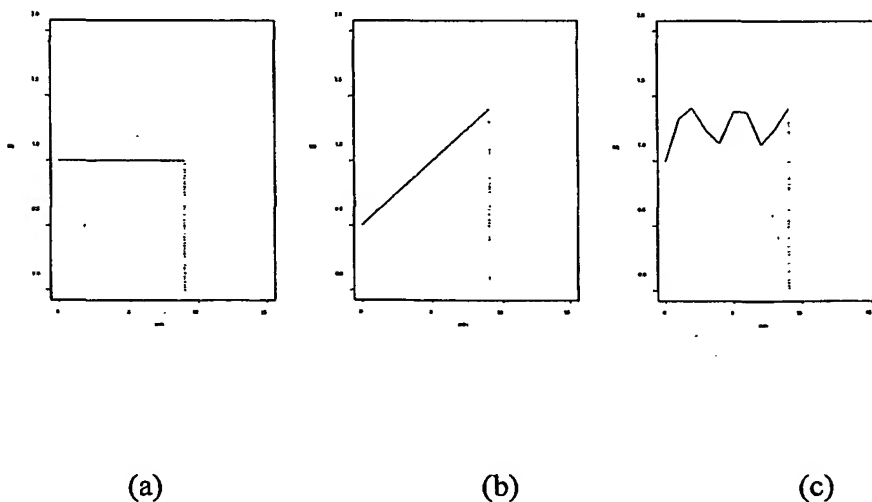
20

The determination of peak widths (base peak widths) was carried out as follows. The peak starting and ending points were defined as the intersection of the tangents at the inflection points of the peak of interest with the baseline of the chromatogram. This corresponds to the peak width at ~ 4.4% peak height. These

points (which define the base peak width under gradient flow conditions) were then converted (through an integration process similar to that described above) to the corresponding peak starting and ending points that would be obtained under isorheic conditions (i.e., 1.0 ml/min). Theoretically, the base peak widths obtained with a

5   gradient flow should be identical to those collected under isorheic conditions.

Mathematica™ (Wolfram Research, Champaign, IL) was used to conduct these calculations. Basically any software package that contains integration functions will be capable of calculating these data.

10   <u>Results from Isorheic and Gradient Flow protocols</u>

A comparison of the chromatographic data (k' and $W_{4.4}$) under isorheic (1.0 ml/min) and gradient flow (ramped gradient from 0.5 ml/min to 4.0 ml/min in 30 min) was initially carried out by collecting the LC/UV chromatograms of a mixture of two compounds (Clozapine and Imipramine) on an $^{ester}IAM.PC^{C10/C3}$ column. The

15   identification of these two peaks (UV absorbance and retention time) was straightforward because of available in-house single compound chromatogram collected under the same isorheic conditions. Very good correlation between gradient flow and isorheic flow data was obtained, and the study was expanded to include 22 additional compounds which were detected and identified by MS.

20   Fig. 4a shows the Total Ion Chromatogram (TIC) and Fig. 4b shows the Extracted Ion Chromatogram (EIC, which is the conversion of the corresponding TIC through a deconvolution process into the individual trace of each ion/compound) of a mixture of 15 compounds under isorheic flow conditions (1.0 ml/min). Figs. 5a-b show the corresponding data under gradient flow conditions.

25   Initial analysis of the data displayed on Figs. 4a-b and 5a-b shows that the gradient flow protocol enhances the resolution of the early eluting peaks. Starting the gradient at a slower flow rate than that used for the isorheic run (i.e.: <1.0ml/min) causes the analytes with weak affinity to elute slower. For example, under isorheic flow conditions Atenolol eluted at 0.53 min., but 0.87 min under gradient flow

30   conditions (Fig. 6a); similarly Minoxidil eluted at 1.26 min. under isorheic conditions, and 2.00 min. under gradient conditions. In essence, the gradient flow method allows

early eluting compounds to be better resolved, and helps minimize the problem of peaks eluting in the dead volume.

On the other hand, because of higher flow rates, gradient flow conditions cause stronger retained compounds to elute out earlier. For instance,

5   diazepam eluted out at 20.14 min. under isorheic flow conditions, as opposed to 14.85 min. with the gradient flow protocol (Fig. 6a); similarly, Delorazepam eluted out more than 10 min. earlier under gradient flow conditions (18.60 instead of 29.53 min.). The value of the gradient flow method is unequivocally demonstrated by the drastic difference in elution time for very strongly retained compounds. For instance,

10 Quazepam and Prochloroperazine (Fig. 6a) eluted at 62.82 and 73.74 min., respectively, under gradient flow conditions, instead of over 4 hours with the isorheic flow protocol. The method considerably reduces data acquisition time and facilitates data analysis since the peaks in chromatograms collected with a flow gradient are in general sharper and taller than those collected at lower flow rates under isorheic

15 conditions. They are thus easier to detect, and accurate peak identification is maximized.

Data Analysis

Correlation plots (24 compounds) between isorheic flow and gradient

20 flow conditions were established for capacity factors (k') (Fig. 6b), peak starting points (Fig. 7b), peak ending points (Fig. 8b) and base peak widths ($W_{4.4}$) (Fig. 9b). Theoretically these two sets of data (isorheic and gradient flow) should be numerically identical. This means that each plot correlating flow gradient parameters with isorheic parameters should have both a slope and an R-squared value (correlation)

25 nearly equal to 1, and an intercept of 0.

Figs. 6a-b through 9a-b unequivocally demonstrate that the chromatographic parameters obtained under gradient flow conditions are very close (virtually identical) to those collected under isorheic conditions. The intercepts from these four plots are close to 0, the slopes are nearly 1 (with less than 0.040 variation),

30 and the R-squared values are almost 1 (with less than 0.0039 variation).

This experimental data confirms that the determination of capacity factors (k') is not affected by the flow rate profile (whether it be constant, stepped,

ramped or a combination thereof) with which chromatographic data are collected: the volume of mobile phase eluted through the column (thus the k' values) at any given time can be derived from an equation describing the flow gradient profile. In addition, we have shown that at least an additional chromatographic parameter (base

5   peak width) can be determined from flow gradient experiments with a great deal of precision. In theory, every single data point on a chromatogram collected under gradient flow conditions can be deconvoluted to constant flow conditions. Conceptually a gradient flow chromatogram can be "converted" point by point to the corresponding constant flow chromatogram, allowing access to the chromatographic

10   parameters (such as peak shape, peak asymmetry, skewness, kurtosis, median, mean, etc.) that are usually sought. In addition, the flow gradient method was shown to enhance resolution for early eluting peaks, and to reduce data acquisition time (instrument time).

       With reference to Fig. 15, there is provided a diagrammatic

15   representation of a chromatographic system in accordance with this invention for determining, storing, and comparing numerical values characteristic of the interaction of one or more test compounds with a surface, a stationary phase, in the chromatographic system. The illustrated system utilizes three chromatographic units, labeled columns 1, 2 and 3. A pump under the control of an electronic control system

20   delivers mobile phase past an injector point and into the chromatographic units. Eluent flow splitters, S_1, S_2, and S_3 are located in fluid flow communication with each of respective columns 1, 2 and 3 and an eluent switch for delivering aliquots of eluent from each of the eluent flow splitters to the detector. The splitters are also in fluid flow communication with a waste collection container through a pressure relief valve

25   (black diamond). Signals from the detector are processed in the electronic control system and directed to an output device or stored in an electronic storage database (memory), typically as a function of retention volume of the eluting/detected compound. Electronic control system includes a microprocessor optionally programmed to retrieve compound data from memory and calculate related values

30   indicative of the interaction of the eluted compounds with the stationary phase on each of the respective columns.

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A routine problem encountered in chromatography is the elution of strongly retained solutes on a surface of interest. Chromatographers often resort to solvent gradients or extrapolation of data obtained at various organic modifier concentrations to circumvent this problem. A study aimed at determining the effect of organic modifier concentration on solute retention was conducted.

A group of 24 drugs were eluted on an ^{ester}IAM.PC$^{C10/C3}$ column with a Phosphate buffer saline (PBS)-based mobile phase containing 0%, 10%, 20%, 30%, and 40% acetonitrile. For each compound, the retention times were plotted against the acetonitrile fraction in the PBS mobile phase. Acetonitrile concentration affects the elution of the solutes in a non-linear way, and that this non-linear dependence is compound specific (different compounds are not affected to the same extent). This organic modifier effect was confirmed, as shown in Figs. 16a-d. The retention times at 0% acetonitrile of a group of 10 of the previous compounds were plotted against the corresponding retention times at 5% (Fig. 16a), 10% (Fig. 16b), 20% (Fig. 16c), and 30% (Fig. 16d) acetonitrile, respectively. Figs. 16a-d clearly show that if there seem to be some correlation for mobile phases that do not differ too much in composition (i.e., 0% ACN vs. 5% ACN), it fades out as the difference in acetonitrile concentration is increased (i.e., 0% ACN vs. 30% ACN).

A group of 10 compounds were eluted under various flow rate conditions. Their respective capacity factors were determined according to the method described previously. The results of the study are represented on Figs. 17a-b through19. Briefly, k' values obtained under isocratic and flow gradient conditions at 10% ACN, 20% ACN, and 30% ACN were compared, and the correlation plots are shown on Figs. 17a, 17b, and 17c, respectively. Excellent correlation was found, with slopes and R-square values approaching 1. Similarly, the k' values of the same 10 compounds eluted with 10% ACN/PBS at 2mL/min, 4 mL/min, and with a flow gradient ramped from 0.5 mL/min to 4 mL/min over 30 minutes were calculated compared to the corresponding k' values obtained at 1 mL/min. The correlation plots are shown on Figs. 18a, 18b, and 18c respectively. The results of the flow gradient study (10% acetonitrile) is summarized on Fig. 19, showing very little variation in the determination of k' values using different flow rate conditions.

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All ten compounds but Etazolate were eluted, using the same flow gradient as mentioned above, at various acetonitrile fractions (0%, 5%, 10%, 20%, and 25%). The capacity factors were calculated according to the method described in this application, and were plotted against acetonitrile concentration (Fig. 20). As

5   observed for isorheic conditions, the dependence of k' with acetonitrile concentration is non-linear and compound specific.

Finally, a study aimed at determining the effect of mobile phase salt concentration (Na_2HPO_4 and NaCl) on solute retention was conducted, and the results are reported on Fig. 21. Salt concentration seems to have minor effects on retention

10   volumes. However, it is worth noting that varying the salt concentration of the mobile phase also affected its pH (Fig. 21). Therefore, the determining factor (salt concentration of pH) responsible for the slight variations in k' is uncertain at this point. Additional studies need to be conducted to elucidate the question.

In conclusion, we have demonstrated that, when the experimental

15   purpose is the determination of chromatographic data characteristic of the interaction of the solutes with the stationary phase, solvent programming (changing the mobile phase composition as a function of time) is not a viable solution for reducing solute retention time. Instead, flow gradient programming is the method of choice.

20   Applications

As described extensively earlier, one example of its application revolves around dependent non-specific binding (as opposed to "specific binding") as a way to differentiate two or more compounds in a test solution. Specific binding is the affinity exhibited between a receptor molecule and a compound wherein the

25   receptor molecule includes a defined binding locus that discriminatorily binds those compounds which have a predetermined chemical structure. Compounds not having the predetermined chemical structure do not bind with the binding site of the receptor molecule. "Compound-dependent non-specific binding" as used herein refers to that affinity interaction between a compound and a surface that does not have a specific

30   discriminative binding locus for that compound, but rather the binding derives from the concomitant hydrophobic and/or hydrophilic interactions between the surface and the compound. Non-specific binding between a surface and a compound is

"compound-dependent" in that, for any one surface, different compounds will interact and bind with such surface to varying degrees based upon the chemical structure and hydrophobic/hydrophilic nature of the compound. The high sensitivity of the MS detector allows the instantaneous identification of mixtures of compounds. A mixture

5   of 100 or more compounds can be injected on the column (or columns run in parallel) and detected as they elute from the chromatographic system. In theory, depending on the loading capacity of the columns, a mixture of up to 1000 compounds can be analyzed. The data from the MS analysis will be correlated to that of the UV detectors connected to the chromatographic system, resulting in the assignment of a

10   retention time and capacity factor for each and every compound detected. The data can be collected electronically and used as input for the calculation of one or more physicochemical values according to predetermined algorithms or equations. In summary, the present invention can be applied to the rapid and efficient collection of databases of physicochemical values and/or biologically relevant parameters for large

15   compound libraries. Consequently it seems perfectly suited for lead identification and optimization of chemical libraries, which is a very important aspect of the drug discovery process, as well as QSAR studies. Once a "hit" compound has been identified, derivatization by the usual combinatorial chemistry tools to a large number of structurally similar parent molecules is possible. The present invention provides a

20   convenient and efficient technique for the analysis of this pool of derivatives and the identification of one or more compounds with a data set of physicochemical values (derived from the chromatographic system) that would classify the compounds of interest as potential promising new leads.

        An obvious example of application of the technique is separation

25   conditions optimization. Since a correlation between the retention volume of a given compound and the gradient flow profile used to elute it can be established, an algorithm similar to that proposed by Jinno et al. can be developed to predict the elution behavior of said compound under various gradient conditions. An initial chromatographic run of a compound mixture under a chosen flow rate profile, coupled

30   with concomitant identification of the eluent, would establish the retention volume for each component in the mixture. This experimental data can then be used to simulate the elution profile of said compound mixture under various flow gradient conditions,

allowing the user to establish the expected flow gradient conditions necessary for optimal resolution and separation of the compounds. Thus it appears that the present invention may be particularly well suited for the study and optimization of separation conditions for any one mixture of compounds.

5   Other examples of applications are drug analysis/screening: evaluation of compounds put in contact with a surface suitable for pharmacokinetic and pharmacodynamic studies. The invention may also find applications in the field of diagnostics: physiological fluids sampling (such as blood or urine) for specific compounds that may be diagnostic of some disease or condition, or for metabolic

10   studies, may be performed. The present invention may also be relevant to environmental sampling (water, soil analyses for contamination) and quality control in the food industry for example (e.g., flavors, ingredients, preservatives, etc.).

CLAIMS:

        1.   A chromatographic system for determining or comparing numerical values characteristic of compound/surface interaction in a sample including a mixture of compounds, said system comprising

        a chromatographic unit having a sample loading port, a mobile phase delivery port, an eluent exit port and a stationary phase;

        a mobile phase supply system including a pump and a source of mobile phase for delivering the mobile phase to the mobile phase delivery port of the chromatographic unit;

        a detector having an eluent sampling port and capable of providing a signal of the presence or identity of a compound in eluent delivered to the sampling port;

        an eluent flow splitter in fluid communication with the eluent exit port and the detector sampling port to minimize variation in flow rate to the detector as a function of rate of flow of mobile phase through the chromatographic unit;

        a programmable controller for the pump for controlling flow rate of mobile phase through the chromatographic unit at a programmed variable flow rate and providing signals indicative of the volume of mobile phase delivered to the chromatographic unit; and

        a data management system in communication with the pump controller and the detector, said data management system comprising a data storage unit for storing signals from the detector as a function of volume of mobile phase delivered to the chromatographic unit.

        2.   The chromatographic system of claim 1 wherein the detector is capable of providing a signal characteristic of the detected compounds.

        3.   The chromatographic system of claim 2 wherein the detector comprises a mass spectrometer, a Fourier transform infra-red spectrometer, a Fourier transform ultraviolet spectrometer or a Fourier transform nuclear magnetic resonance spectrometer.

        4.   The chromatographic system of claim 2 wherein the detector comprises a mass spectrometer.

5.   The chromatographic system of claim 1 wherein the detector is a mass spectrometer and the data management system includes a data storage unit for storing signals indicative of the mass of detected compounds as a function of the volume of the mobile phase delivered to the chromatographic unit.

6.   The chromatographic system of claim 5 wherein the splitter includes a chamber having an eluent inlet and two eluent outlets, one in fluid flow communication with a waste collection vessel through a pressure relief valve and the other in fluid flow communication with the sampling port of the detector.

7.   The chromatographic system of claim 1 comprising at least two chromatographic units.

8.   The chromatographic system of claim 7 further comprising eluent flow splitters in fluid communication with the eluent exit port of each chromatographic unit, each of said eluent flow splitters comprising a chamber having an eluent inlet and two eluent outlets, one in fluid flow communication with a fluid collection vessel through a pressure relief valve and the other in fluid flow communication with the detector sampling port through a valve for receiving eluent from each of the splitters and delivering aliquots of eluent from each of the eluent flow splitters to the sampling port of the detector.

9.   The chromatographic system of claim 7 further comprising eluent flow splitters in fluid communication with the eluent exit port of each chromatographic unit, each of said eluent flow splitters comprising a chamber having an eluent inlet and two eluent outlets, one in fluid flow communication with a fluid collection vessel through a pressure relief device and the other in fluid flow communication with a detector sampling port.

10.   The chromatographic system of claim 1 further comprising a display device in communication with the data management system for reporting the detector signal as a function of retention volume.

11.   The chromatographic system of claim 1 wherein the data management system further comprises a microprocessor programmed to calculate and store k' values for at least a portion of the eluted compounds wherein $k' = (V_r-V_o)/V_o$ wherein V_r is the retention volume for an eluted compound and V_o is the dead volume of the chromatographic unit.

12.   The chromatographic system of claim 1 further comprising a data output device for reporting the k' value $(V_r-V_o)/V_o$ wherein V_r is the retention volume and V_o is the void volume of the chromatographic unit) for at least a portion of the compounds eluted from the chromatographic unit.

13.   The chromatographic system of claim 1 wherein the detector is a mass spectrometer providing a signal of total ion concentration and the data management system further comprises an algorithm for identifying the signals from the detector for the individual compounds detected to produce an extracted ion chromatogram as a function of retention volume or retention time.

14.   The chromatographic system of claim 1 wherein the controller for the pump is programmed to modulate the flow rate of mobile phase through the chromatographic unit according to a predetermined flow rate profile.

15.   The chromatographic system of claim 14 wherein the flow rate profile can be represented by the formula flow rate = a + bt wherein a is the initial flow rate, t is the time after sample delivery, and b is the slope of the plot of flow rate vs. time.

16.   The chromatographic system of claim 14 wherein the flow rate profile is stepped.

17.   The chromatographic system of claim 1 wherein the stationary phase comprises an immobilized artificial membrane or an immobilized macromolecule.

18.   In a method for determining or comparing numerical values characteristic of compound/surface interaction of compounds in a mixture of compounds, which method comprises the step of subjecting the mixture of compounds to a chromatographic separation in a chromatographic unit comprising a stationary phase and a mobile phase, the improvement comprising the step of carrying out the chromatographic separation in a chromatographic system comprising

a chromatographic unit having a sample loading port, a mobile phase delivery port, an eluent exit port and a stationary phase;

a mobile phase supply system including a pump and a source of mobile phase for delivering the mobile phase to the mobile phase delivery port of the chromatographic unit;

a detector having an eluent sampling port and capable of providing a signal of the presence or identity of a compound in eluent delivered to the sampling port;

an eluent flow splitter in fluid communication with the eluent exit port and the detector sampling port to minimize variation in flow rate to the detector as a function of rate of flow of mobile phase through the chromatographic unit;

a programmable controller for the pump for controlling flow rate of mobile phase through the chromatographic unit at a programmed variable flow rate and providing signals indicative of the volume of mobile phase delivered to the chromatographic unit; and

a data management system in communication with the pump controller and the detector, said data management system comprising a data storage unit for storing signals form the detector as a function of volume of mobile phase delivered to the chromatographic unit.

19.   In a method of screening test compounds for biological properties comprising the steps of combining said test compounds with a training set composition comprising one or more control compounds having a common biological property to provide a test mixture and subjecting at least a portion of said mixture to chromatographic separation in a chromatographic system comprising a stationary phase and a mobile phase to define numerical values characteristic of the interaction of the compounds in the test mixture with the stationary phase and comparing said numerical values to identify test compounds having numerical values that best match the numerical values for the control compounds, the improvement comprising the steps of carrying out the chromatographic separation in a chromatographic system comprising

a chromatographic unit having a sample loading port, a mobile phase delivery port, an eluent exit port and a stationary phase;

a mobile phase supply system including a pump and a source of mobile phase for delivering the mobile phase to the mobile phase delivery port of the chromatographic unit;

a detector having an eluent sampling port and capable of providing a
signal of the presence or identity of a compound in eluent delivered to the sampling
port;

an eluent flow splitter in fluid communication with the eluent exit port

5 and the detector sampling port to minimize variation in flow rate to the detector as a
function of rate of flow of mobile phase through the chromatographic unit;

a programmable controller for the pump for controlling flow rate of
mobile phase through the chromatographic unit at a programmed flow rate and
providing signals indicative of the volume of mobile phase delivered to the

10 chromatographic unit; and

a data management system in communication with the pump controller
and the detector, said data management system comprising a data storage unit for
storing signals from the detector as a function of volume of mobile phase delivered to
column,

15 wherein said numerical values characteristic of the interaction of each
compound with the stationary phase is the value $k' = (V_r-V_o)/V_o$ for each compound
wherein V_r is the retention volume for said compound and V_o is the dead volume of
the chromatographic unit.

20. The improved method of claim 18 or claim 19 wherein the

20 stationary phase comprises an immobilized artificial membrane or an immobilized
macrmolecule.

21. A method for creating a database of numerical values
characteristic of the interaction of solute test compounds with a mobile phase and a
stationary phase in at least two chromatographic separation systems, said method

25 comprising the steps of

selecting a mobile phase, a stationary phase, and an initial mobile flow
rate independently for each chromatographic separation system;

delivering one or more test compounds to the chromatographic
separation systems for separation in said systems;

30 changing the flow rate of the mobile phase during separation of the
compounds in at least one chromatographic separation system; and

detecting at least a subset of the compounds as they elute from each system, and

storing numerical values indicative of the elution volume, the elution peak profile, or a value derived from said values for each detected compound in an electronic database.

22.   The method of claim 21 wherein the eluted compounds are subjected to spectral analysis as they are eluted from the system, and numerical values derived from said spectral analysis are stored as a function of the retention volume for the eluted compounds in the electronic storage device.

23.   The method of claim 21 wherein the numerical values characteristic of the interaction of the eluted solute compounds between the mobile phase and the stationary phase comprise the respective retention volume of the eluted compounds or values derived from algorithmic manipulation of data relating to elution peak profiles, said values selected from the group consisting of capacity factor, peak width, standard deviations, peak skewness, peak asymmetry, peak kurtosis, and other properties that can be calculated from chromatographic peak data.

24.   The method of claim 22 wherein the numerical values characteristic of the interaction of the eluted solute compounds between the mobile phase and the stationary phase comprise the retention volume of the compounds, the elution peak profile or other numeric values derived from algorithmic manipulation of data relating to elution peak profiles selected from the group consisting of capacity factor, peak width, standard deviations, peak skewness, peak asymmetry, peak kurtosis, and other properties that can be calculated from chromatographic peak data.

25.   The method of claim 21 wherein the numerical values are representative of solute pKa properties in which method the steps are repeated using mobile phases of unique pH.

26.   The method of claim 21 wherein the numerical values are representative of solute thermodynamic properties in which method the steps are repeated while maintaining at least one chromatographic system at unique temperatures.

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27.   The method of claim 21 wherein the numerical values are representative of solute properties in which method the steps are repeated using mobile phases of unique ionic strength.

28.   The method of claim 21 wherein the flow rate is increased during data acquisition to obtain numerical values for multiple compounds from a single injection.

29.   The method of claim 21 further comprising the step of collecting fractions of at least a portion of the compounds eluting from the chromatographic system.

30.   The method of claim 22 further comprising the step of collecting fractions of at least a portion of the compounds eluting from the chromatographic system.

31.   The method of claim 28 further comprising the step of collecting fractions of at least a portion of the compounds eluting from the chromatographic system.

32.   The method of claim 21 further comprising the step of analyzing the fractions for a predetermined biological activity.

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- PROCAINE
▲ MEPIVACAINE
▼ BUPIVACAINE
◆ LIDOCAINE
+ PROPRANOLOL
✕ ALPRENOLOL

✳ TOCAINIDE
– W36017
I PRILOCAINE
□ GEA 968
○ ETIDOCAINE
△ TETRACAINE
▽ TRIMECAINE

Log k'_IAM

MAF 10%

MAF 20%

MAF 25%

MAF 30%

φ

FIG. 1

IAM 2

A [1,3,2]

B [2,1,2]

IAM 3

IAM 1

FIG. 2a

B [2,3,2]

A [1,2,2]

IAM 3

FIG. 2b

IAM 1

PRESSURE
RELEASE
VALVE

FLOW TO A
WASTE BOTTLE
INCREASES

FLOW FROM
COLUMN
INCREASES

FLOW TO A
DETECTOR
IS CONSTANT

COLUMN

INJECTOR

PUMPING
SYSTEM

FIG. 3

FIG 4 a

FIG. 4 b
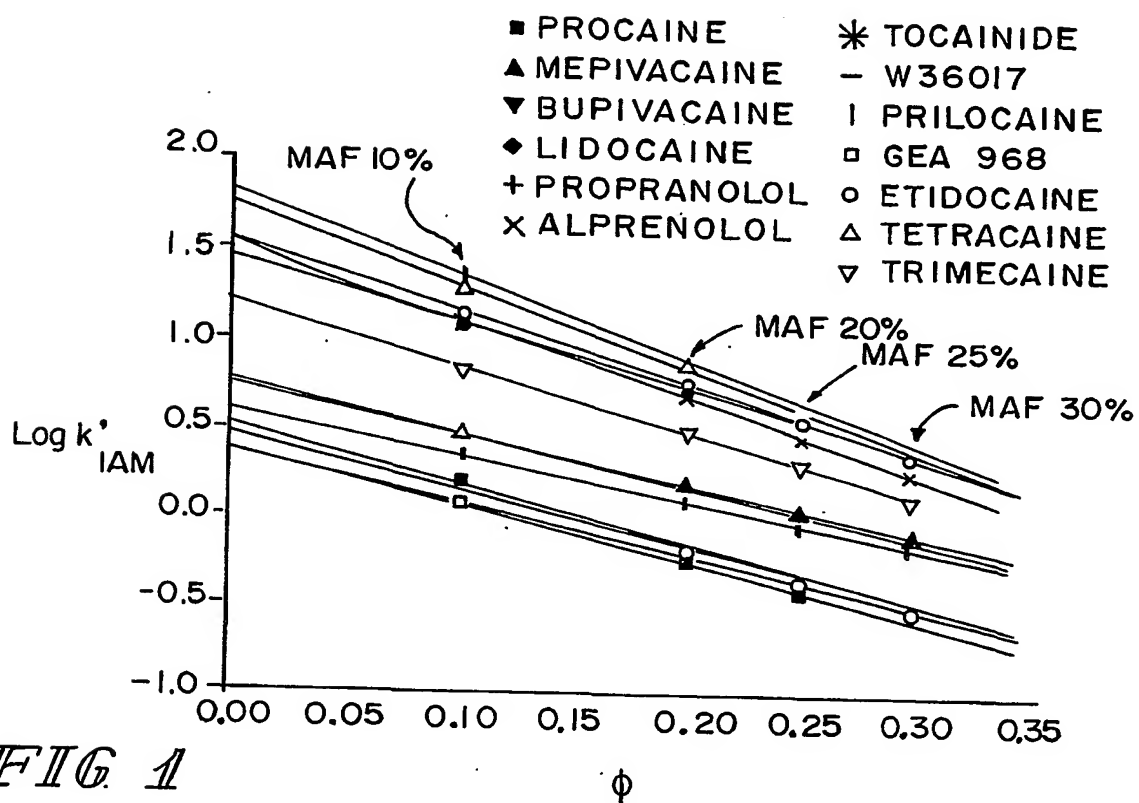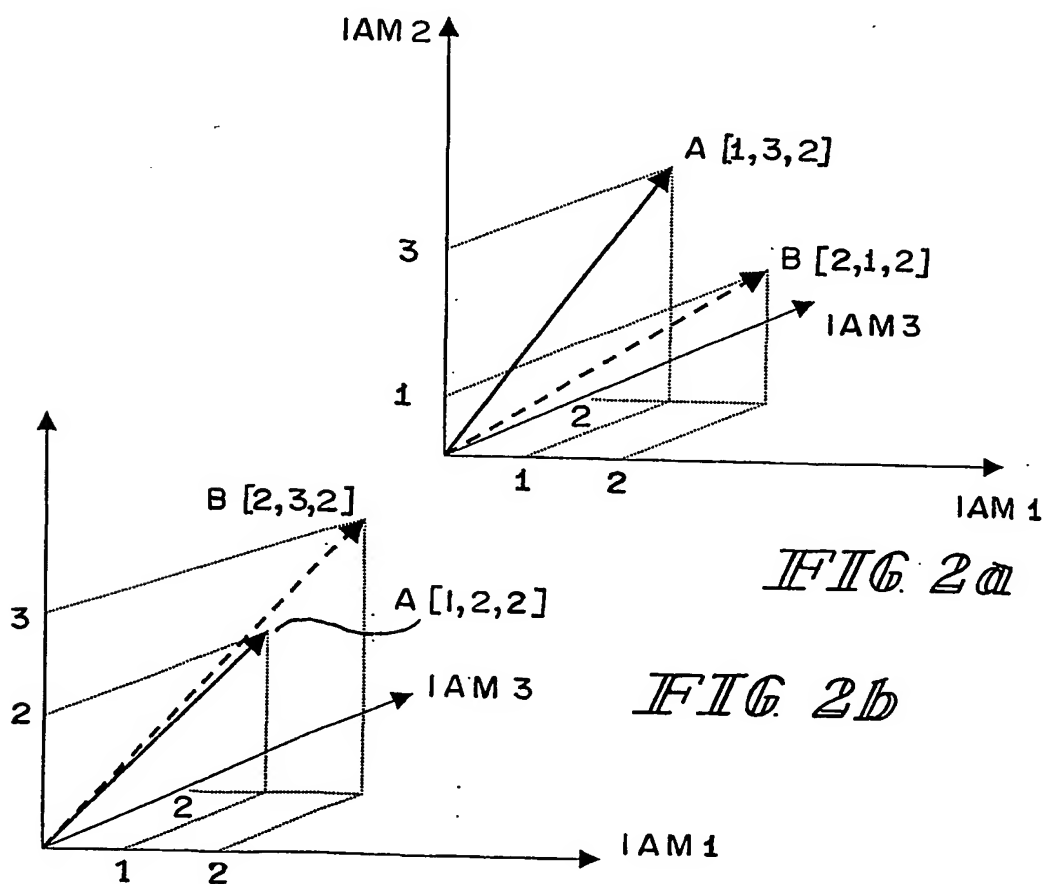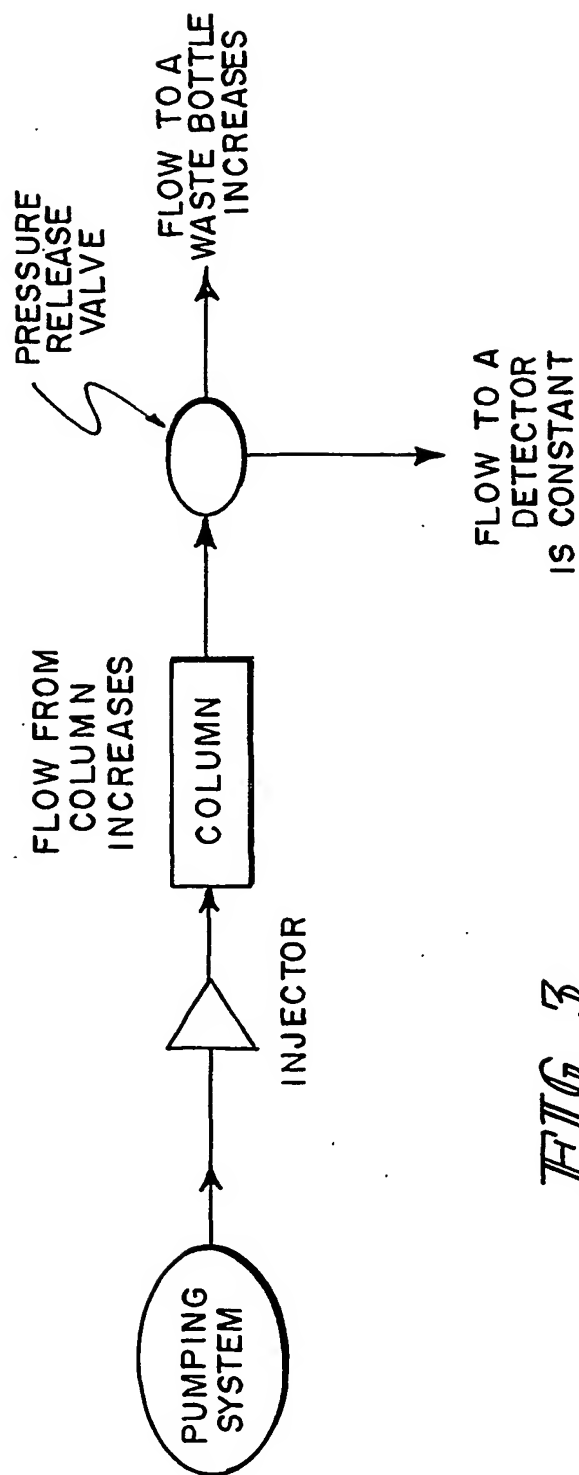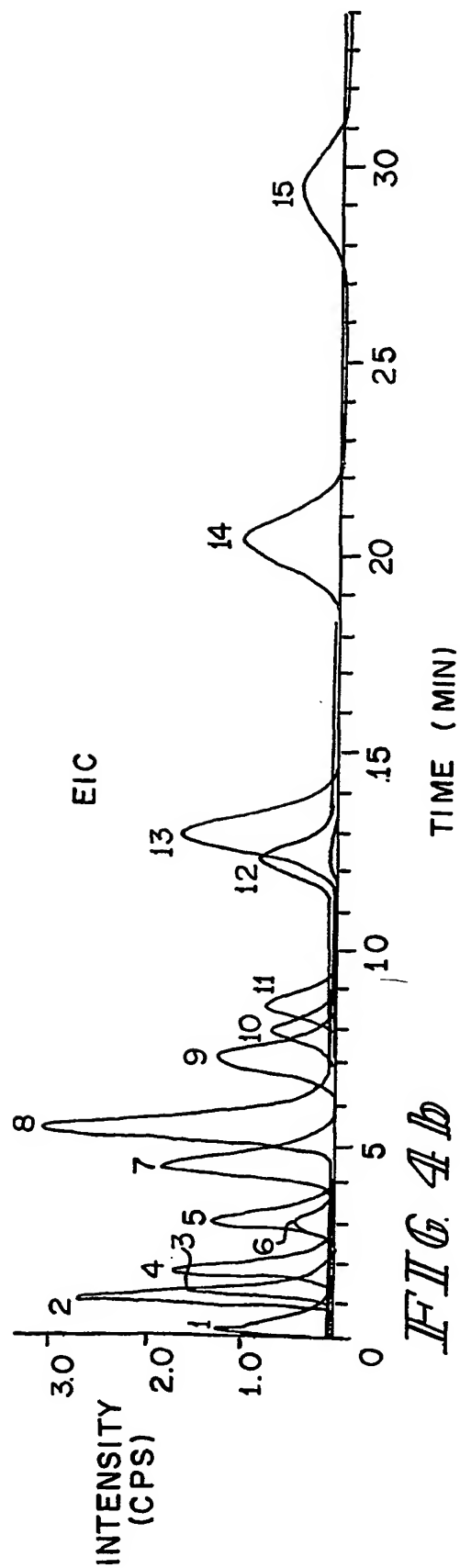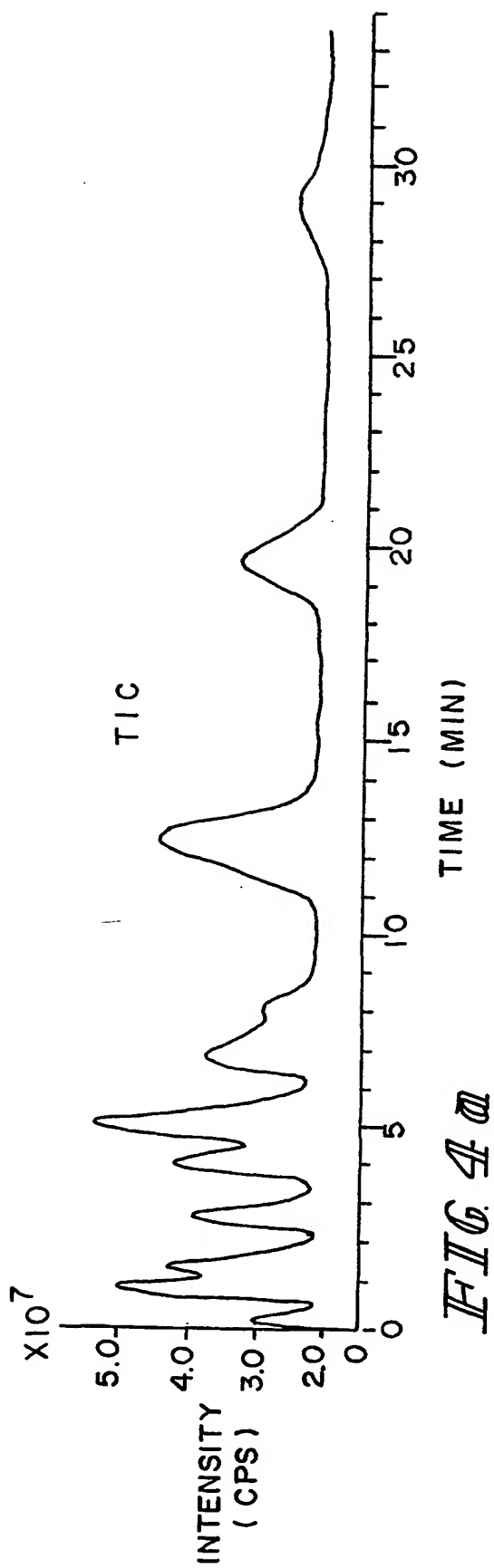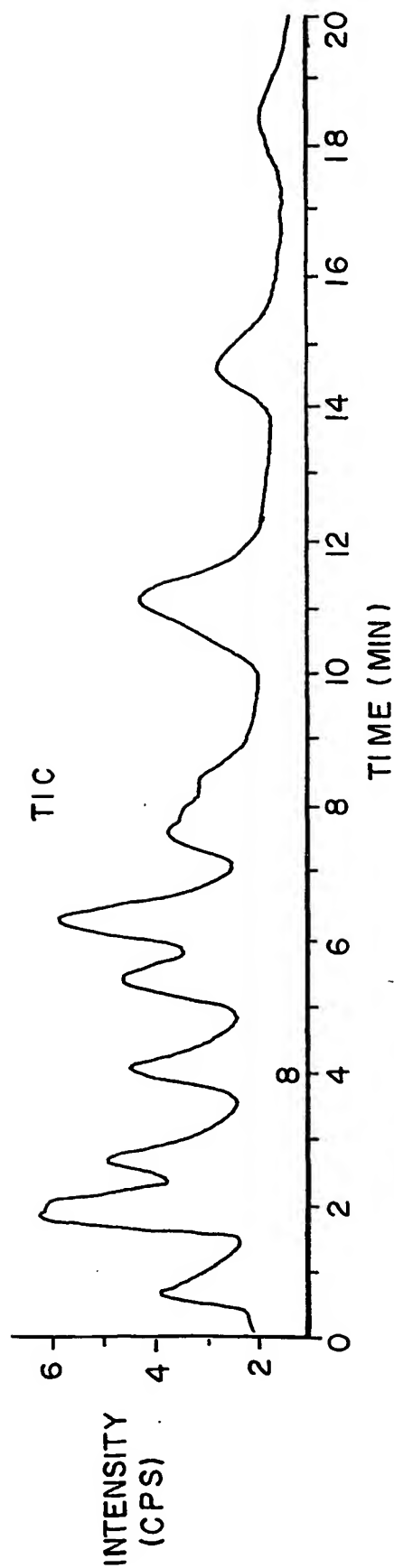
FIG. 5a
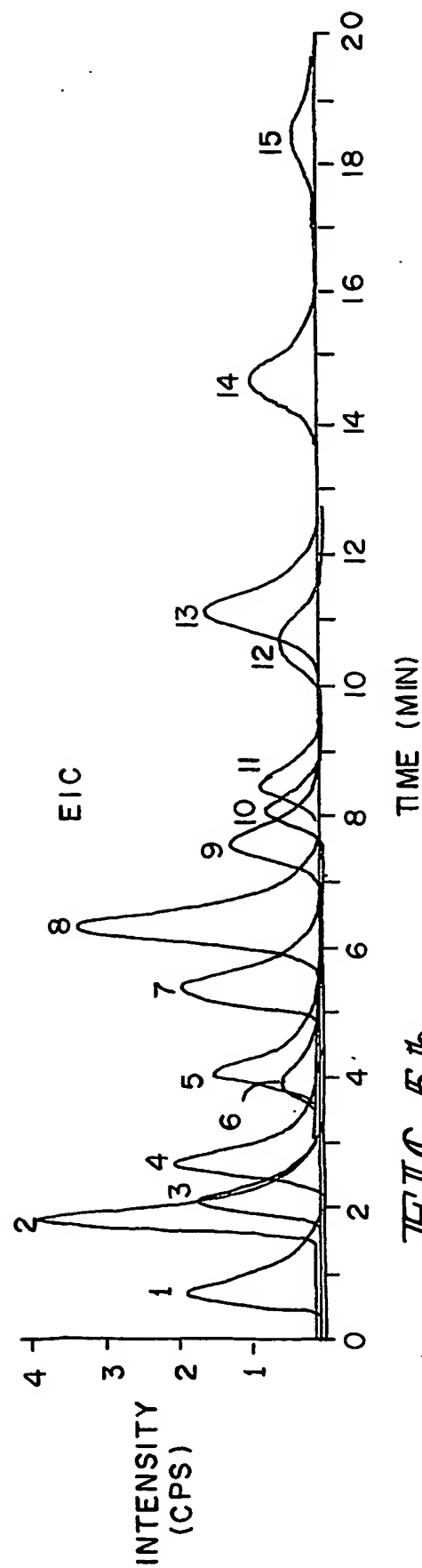
FIG. 5b

ITEM	COMPOUND NAME	ISORHEIC FLOW (T_0 = 0.42MIN)		GRADIENT FLOW (V_0 = 0.39ML)		
		TR (MIN)	K'	PEAK TIME (MIN)	VR (ML)	K'
1	ATENOLOL	0.53	0.39	0.87	0.48	0.23
2	MINOXIDIL	1.26	2.32	2.00	1.24	2.16
3	INDOPROFEN	1.48	2.89	2.27	1.44	2.68
4	ACEBUTOLOL	1.93	4.18	2.90	1.94	3.98
5	NALBUPHINE	3.19	7.39	4.25	3.18	7.15
6	MINAPRINE	3.19	7.39	4.16	3.09	6.93
7	8-0H-DPAT	4.59	11.07	5.60	4.63	10.87
8	ESTAZOLAM	5.66	13.89	6.50	5.72	13.66
9	BUSPIRONE	7.38	18.42	7.76	7.40	17.97
10	FLUNITRAZEPAM	8.05	20.18	8.25	8.11	19.77
11	CYHEPTAMIDE	8.68	21.84	8.66	8.72	21.33
12	CHLORDIAZEPOXIDE	12.11	30.87	10.82	12.26	30.41
13	TRAZODONE	13.01	33.24	11.36	13.23	32.89
14	DIAZEPAM	20.14	52.00	14.85	20.33	51.07
15	DELORAZEPAM	29.53	76.71	18.60	29.54	74.68
16	CLOZAPINE	6.75	17.5	7.38	6.88	17.88
17	IMIPRAMINE	12.28	32.73	10.95	12.49	33.27
18	PROMETHAZINE	41.29	99.95	22.41	40.58	101.44
19	CHLOROPROMAZINE	84.74	205.68	33.65	82.25	206.70
20	IPRINDOLE	84.87	206.00	34.01	83.69	210.34
21	CINNARIZINE	115.89	281.66	40.60	110.05	276.90
22	TRIFLUPROMAZINE	116.24	282.51	40.87	111.13	279.63
23	QUAZEPAM	207.26	504.51	62.82	198.93	501.35
24	PROCHLOROPERAZINE	247.90	603.63	73.74	242.61	611.65

FIG. 6a

ITEM	COMPOUND NAME	ISORHEIC FLOW ($T_0=0.42$MIN) PEAK START (MIN)	GRADIENT FLOW ($V_0=0.39$ML) PEAK START (MIN)	NORMALIZED PEAK START (MIN)
1	ATENOLOL	0.35	0.54	0.29
2	MINOXIDIL	0.95	1.64	0.98
3	INDOPROFEN	1.15	1.94	1.19
4	ACEBUTOLOL	1.55	2.44	1.57
5	NALBUPHINE	2.65	3.74	2.69
6	MINAPRINE	2.55	3.64	2.6
7	8-OH-DPAT	3.75	4.94	3.9
8	ESTAZOLAM	4.55	5.64	4.68
9	BUSPIRONE	6.15	6.94	6.29
10	FLUNITRAZEPAM	7.15	7.54	7.1
11	CYHEPTAMIDE	7.75	7.94	7.66
12	CHLORDIAZEPOXIDE	10.85	9.94	10.75
13	TRAZODONE	11.45	10.24	11.25
14	DIAZEPAM	18.15	13.74	17.91
15	DELORAZEPAM	26.65	17.24	26.01
16	CLOZAPINE	6.33	6.90	6.24
17	IMIPRAMINE	11.64	10.30	11.36
18	PROMETHAZINE	37.86	20.92	36.06
19	CHLOROPROMAZINE	79.2	31.82	74.93
20	IPRINDOLE	79.76	32.12	76.13
21	CINNARIZINE	99.36	36.32	92.93
22	TRIFLUPROMAZINE	95.86	36.02	91.73
23	QUAZEPAM	142.86	49.32	144.93
24	PROCHLOROPERAZINE	238.46	69.72	226.53

FIG. 1a

GRADIENT FLOW k'
(0.5 TO 4.0 mL/min
15 % ACETONITRILE
IN NH$_4$OAc OVER
30 min)

$y = 1.0024x$
$R^2 = 0.9998$

ISORHEIC FLOW k'
(1 ml/min 15% ACETONITRILE IN NH$_4$OAc)

FIG. 6b

GRADIENT FLOW
PEAK STARTING
POINT
(0.5 TO 4.0 mL/min
15% ACETONITRILE
IN NH$_4$OAc OVER
30 min)

$y = 0.9616x$
$R^2 = 0.999$

ISORHEIC FLOW PEAK STARTING POINTS
(1 mL/min 15% ACETONITRILE IN NH$_4$OAc)
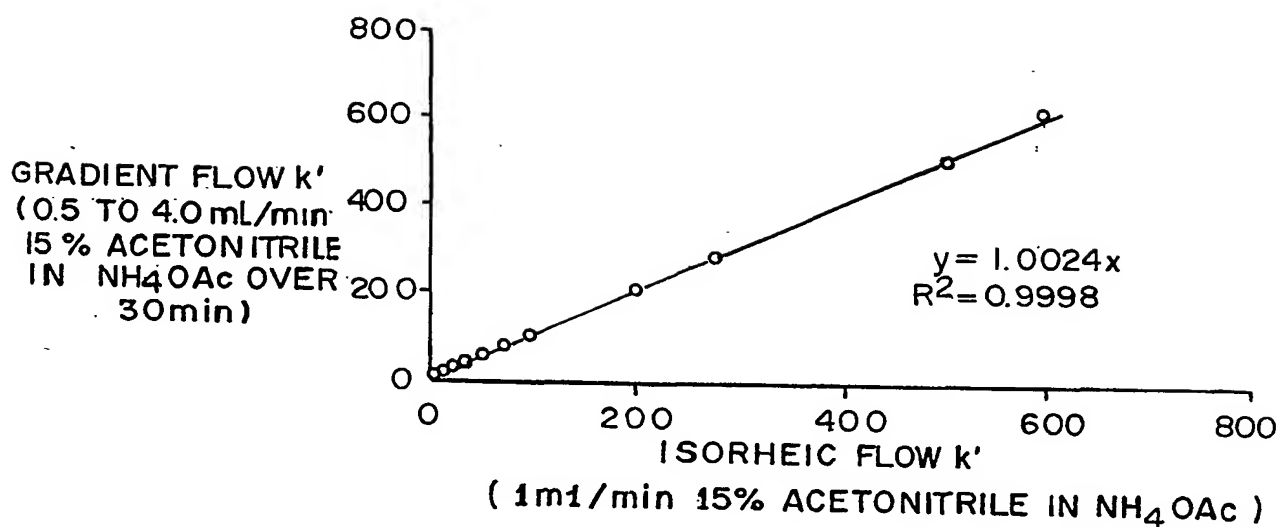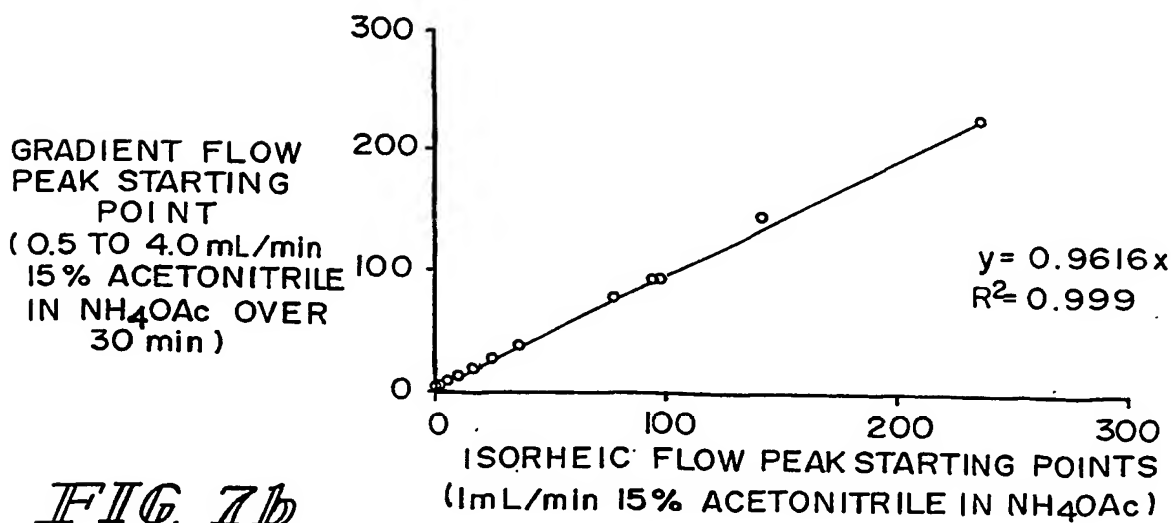
FIG. 7b

ITEM	COMPOUND NAME	ISORHEIC FLOW (T_0= 0.42MIN) PEAK END (MIN)	GRADIENT FLOW (V_0= 0.39ML)	
			PEAK END (MIN)	NORMALIZED PEAK END (MIN)
1	ATENOLOL	1.75	2.34	1.49
2	MINOXIDIL	2.95	3.54	2.50
3	INDOPROFEN	3.15	3.74	2.69
4	ACEBUTOLOL	3.45	4.34	3.27
5	NALBUPHINE	5.25	5.64	4.68
6	MINAPRINE	4.75	5.64	4.68
7	8-0H-DPAT	6.75	8.04	7.80
8	ESTAZOLAM	8.25	8.84	8.99
9	BUSPIRONE	8.85	9.34	9.77
10	FLUNITRAZEPAM	9.45	9.64	10.26
11	CYHEPTAMIDE	10.35	10.24	11.25
12	CHLORDIAZEPOXIDE	13.85	12.34	15.08
13	TRAZODONE	15.15	13.14	16.67
14	DIAZEPAM	22.55	16.44	24.03
15	DELORAZEPAM	32.35	20.34	34.37
16	CLOZAPINE	8.30	8.35	8.25
17	IMIPRAMINE	14.80	12.30	15.00
18	PROMETHAZINE	52.06	26.22	53.33
19	CHLOROPROMAZINE	105.50	38.62	102.13
20	IPRINDOLE	105.96	39.52	105.73
21	CINNARIZINE	134.86	46.22	132.53
22	TRIFLUPROMAZINE	142.16	48.42	141.33
23	QUAZEPAM	224.76	69.92	227.33
24	PROCHLOROPERAZINE	320.26	90.22	308.53

FIG. 8a

ITEM	COMPOUND NAME	ISORHEIC FLOW (T_0 = 0.42MIN) PEAK WIDTH	GRADIENT FLOW (V_0 = 0.39ML) NORMALIZED PEAK WIDTH
1	ATENOLOL	1.40	1.20
2	MINOXIDIL	2.00	1.52
3	INDOPROFEN	2.00	1.50
4	ACEBUTOLOL	1.90	1.70
5	NALBUPHINE	2.60	1.99
6	MINAPRINE	2.20	2.08
7	8-0H-DPAT	3.00	3.90
8	ESTAZOLAM	3.70	4.31
9	BUSPIRONE	2.70	3.48
10	FLUNITRAZEPAM	2.30	3.16
11	CYHEPTAMIDE	2.60	3.59
12	CHLORDIAZEPOXIDE	3.00	4.33
13	TRAZODONE	3.70	5.42
14	DIAZEPAM	4.40	6.12
15	DELORAZEPAM	5.70	8.36
16	CLOZAPINE	1.97	2.01
17	IMIPRAMINE	3.16	3.64
18	PROMETHAZINE	14.20	17.27
19	CHLOROPROMAZINE	26.30	27.20
20	IPRINDOLE	26.20	29.60
21	CINNARIZINE	35.50	39.60
22	TRIFLUPROMAZINE	46.30	49.60
23	QUAZEPAM	81.90	82.40
24	PROCHLOROPERAZINE	81.80	82.00

FIG. 9a

GRADIENT FLOW
PEAK ENDING
POINTS
(0.5 TO 4.0 mL/min
15 % ACETONITRILE
IN NH₄OAc OVER
30 min)

$y = 0.9829x$
$R^2 = 0.9992$

ISORHEIC FLOW PEAK ENDING POINTS

$FIG. 8b$ (1mL/min 15% ACETONITRILE IN NH₄OAc)

GRADIENT
FLOW 5xσ_{SD}
(0.5 TO 4.0 mL/min
15 % ACETONITRILE
IN NH₄OAc OVER
30 min)

$y = 1.0299x$
$R^2 = 0.9961$

ISORHEIC FLOW 5xσ_{SD}

$FIG. 9b$ (1mL/min 15% ACETONITRILE IN NH₄OAc)
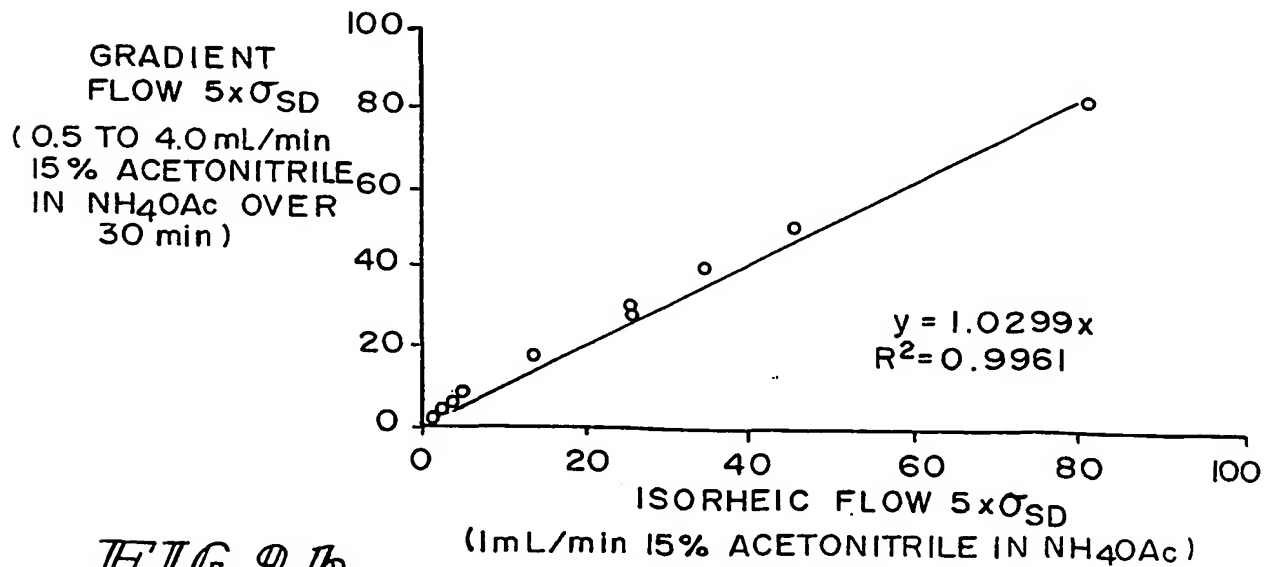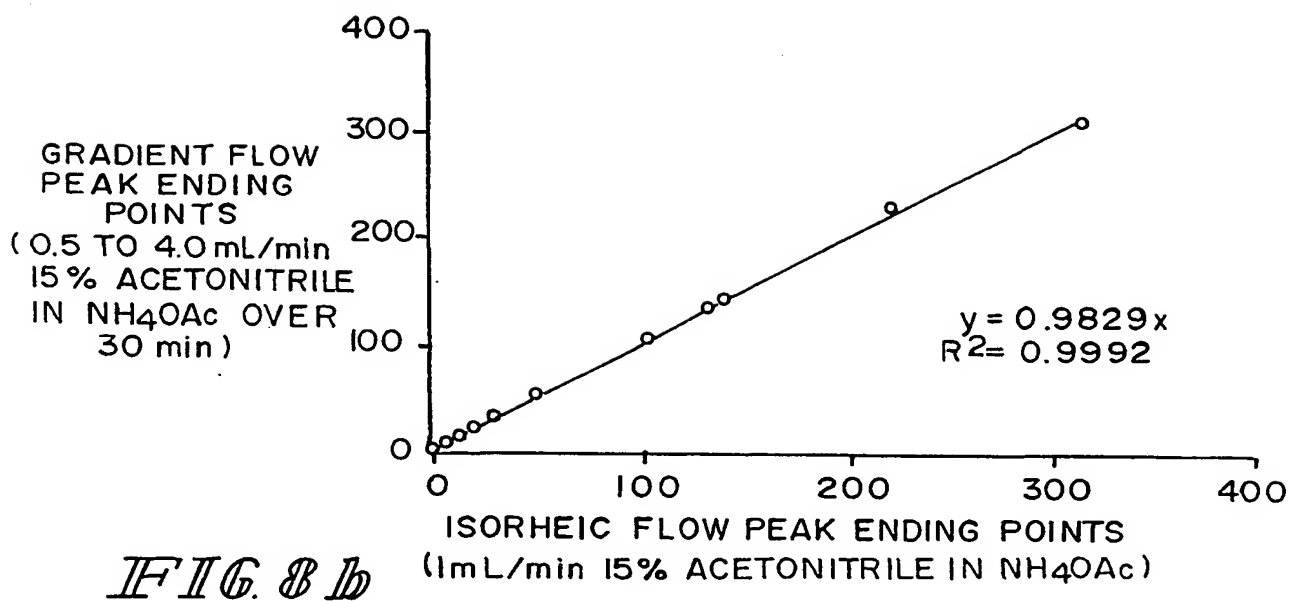
ITEM	COMPOUND NAME	ISOCRATIC ACN (T$_0$= 0.42MIN)		GRADIENT ACN (T$_0$ = 0.48MIN)	
		TR (MIN)	K'	TR (MIN)	K'
1	ATENOLOL	0.53	0.39	0.85	0.77
2	MINOXIDIL	1.26	2.32	3.10	5.46
3	INDOPROFEN	1.48	2.89	8.40	16.50
4	ACEBUTOLOL	1.93	4.18	3.50	6.29
5	NALBUPHINE	3.19	7.39	5.34	10.13
6	MINAPRINE	3.19	7.39	11.26	22.46
7	8-0H-DPAT	4.59	11.07	6.96	13.50
8	ESTAZOLAM	5.66	13.89	13.87	27.90
9	BUSPIRONE	7.38	18.42	13.33	26.77
10	FLUNITRAZEPAM	8.05	20.18	14.32	28.83
11	CYHEPTAMIDE	8.68	21.84	14.76	29.75
12	CHLORDIAZEPOXIDE	12.11	30.87	17.49	35.44
13	TRAZODONE	13.01	33.24	18.12	36.75
14	DIAZEPAM	20.14	52.00	20.98	42.71
15	DELORAZEPAM	29.53	76.71	23.63	48.23
16	CLOZAPINE	6.75	17.50	28.55	58.48
17	IMIPRAMINE	12.28	32.73	22.96	46.83

FIG. 10 a

ITEM	COMPOUND NAME	ISOCRATIC ACN (T = 0.42MIN) PEAK START (MIN)	GRADIENT ACN (T = 0.48MIN) PEAK START (MIN)
1	ATENOLOL	0.35	0.30
2	MINOXIDIL	0.95	2.50
3	INDOPROFEN	1.25	2.80
4	ACEBUTOLOL	1.65	4.50
5	NALBUPHINE	2.75	6.10
6	MINAPRINE	2.75	7.60
7	8-0H-DPAT	4.05	10.50
8	ESTAZOLAM	4.95	12.50
9	BUSPIRONE	6.65	13.10
10	FLUNITRAZEPAM	7.35	13.70
11	CYHEPTAMIDE	8.05	14.20
12	CHLORDIAZEPOXIDE	11.25	16.80
13	TRAZODONE	11.85	17.40
14	DIAZEPAM	18.75	20.10
15	DELORAZEPAM	27.55	22.20
16	CLOZAPINE	6.33	23.00
17	IMIPRAMINE	11.64	27.70

FIG. 11a

GRADIENT
ACETONITRILE k'
(5% TO 30%
ACETONITRILE
IN NH$_4$OAc
OVER 30min)

$y = 0.6114x + 14.176$
$R^2 = 0.5367$

ISOCRATIC ACETONITRILE
(15% ACETONITRILE IN NH$_4$OAc)

FIG. 10 b

PEAK STARTING
POINTS FROM
GRADIENT
ACETONITRILE
ELUTION
(5% TO 30%
ACETONITRILE
IN NH$_4$OAc
OVER 30min)

$y = 0.8485x + 6.252$
$R^2 = 0.5755$

PEAK STARTING POINTS FROM ISOCRATIC
ACETONITRILE ELUTION
(15% ACETONITRILE IN NH$_4$OAc)

FIG. 11 b

ITEM	COMPOUND NAME	ISOCRATIC ACN (T_0 = 0.42MIN) PEAK END (MIN)	GRADIENT ACN (T_0 = 0.48MIN) PEAK END (MIN)
1	ATENOLOL	1.35	1.70
2	MINOXIDIL	2.25	4.10
3	INDOPROFEN	2.45	4.60
4	ACEBUTOLOL	2.95	6.40
5	NALBUPHINE	4.15	8.00
6	MINAPRINE	4.05	9.20
7	8-0H-DPAT	5.75	12.30
8	ESTAZOLAM	7.05	14.40
9	BUSPIRONE	8.65	14.80
10	FLUNITRAZEPAM	8.95	14.80
11	CYHEPTAMIDE	9.55	15.50
12	CHLORDIAZEPOXIDE	13.25	18.20
13	TRAZODONE	14.45	19.00
14	DIAZEPAM	21.55	22.40
15	DELORAZEPAM	31.75	24.30
16	CLOZAPINE	8.30	24.30
17	IMIPRAMINE	14.80	30.10

FIG. 12a

ITEM	COMPOUND NAME	ISOCRATIC ACN (T_0 = 0.42MIN) PEAK WIDTH	GRADIENT ACN (T_0 = 0.48MIN) PEAK WIDTH
1	ATENOLOL	1.00	1.40
2	MINOXIDIL	1.30	1.60
3	INDOPROFEN	1.20	1.80
4	ACEBUTOLOL	1.30	1.90
5	NALBUPHINE	1.40	1.90
6	MINAPRINE	1.30	1.60
7	8-0H-DPAT	1.70	1.80
8	ESTAZOLAM	2.10	1.90
9	BUSPIRONE	2.00	1.70
10	FLUNITRAZEPAM	1.60	1.10
11	CYHEPTAMIDE	1.50	1.30
12	CHLORDIAZEPOXIDE	2.00	1.40
13	TRAZODONE	2.60	1.60
14	DIAZEPAM	2.80	2.30
15	DELORAZEPAM	4.20	2.10
16	CLOZAPINE	1.97	1.30
17	IMIPRAMINE	3.16	2.40

FIG. 13a

PEAK ENDING
POINTS FROM
GRADIENT
ACETONITRILE
ELUTION

(5% TO 30%
ACETONITRILE
IN NH$_4$OAc
OVER 30min)

$y = 1.5799x + 0.1397$
$R^2 = 0.976$

PEAK ENDING POINTS FROM ISOCRATIC
ACETONITRILE ELUTION
(15% ACETONITRILE IN NH$_4$OAc)

FIG. 12b

ACETONITRILE
GRADIENT 5xσ_{SD}

(5% TO 30%
ACETONITRILE
IN NH$_4$OAc
OVER 30min)

$y = 0.2395x + 1.2449$
$R^2 = 0.3132$

ISOCRATIC ACETONITRILE 5xσ_{SD}
(15% ACETONITRILE IN NH$_4$OAc)

FIG. 13b

FIG. 14

FIG. 15

5% ACN vs 0% ACN

$y = 0.3622x + 11.094$

$R^2 = 0.9417$

5% ACN (y-axis) vs **0% ACN** (x-axis)

FIG. 16a

10% ACN vs 0% ACN

$y = 0.249x + 4.8658$

$R^2 = 0.9093$

10% ACN (y-axis) vs **0% ACN** (x-axis)

FIG. 16b

20% ACN vs 0% ACN

$y = 0.0416x + 3.2595$

$R^2 = 0.7243$

FIG. 16c

30% ACN vs 0% ACN

$y = 0.0094x + 1.5523$

$R^2 = 0.5782$

FIG. 16d

10% ACN

$y = 0.9807x + 0.7713$

$R^2 = 0.9994$

FIG. 17a

20% ACN

$y = 1.2107x - 1.4935$

$R^2 = 0.9527$

FIG. 17b

30% ACN

$y = 0.9963x + 0.3456$

$R^2 = 0.9959$

FIG. 17c

k' (2mL/min
ISORHEIC)

$$y = 0.9722x + 0.6637$$
$$R^2 = 0.9995$$

k' ISORHEIC FLOW (1mL/min)

FIG. 18a

k' (4 mL/min
ISORHEIC)
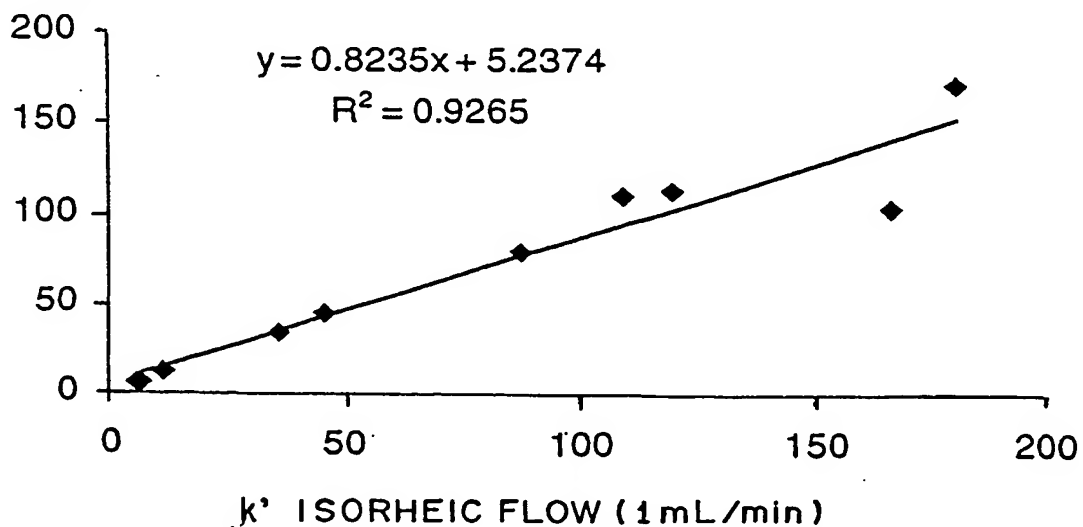
$$y = 0.8235x + 5.2374$$
$$R^2 = 0.9265$$

k' ISORHEIC FLOW (1mL/min)

FIG. 18b

FIG. 18c

Flowrate (mL/min)
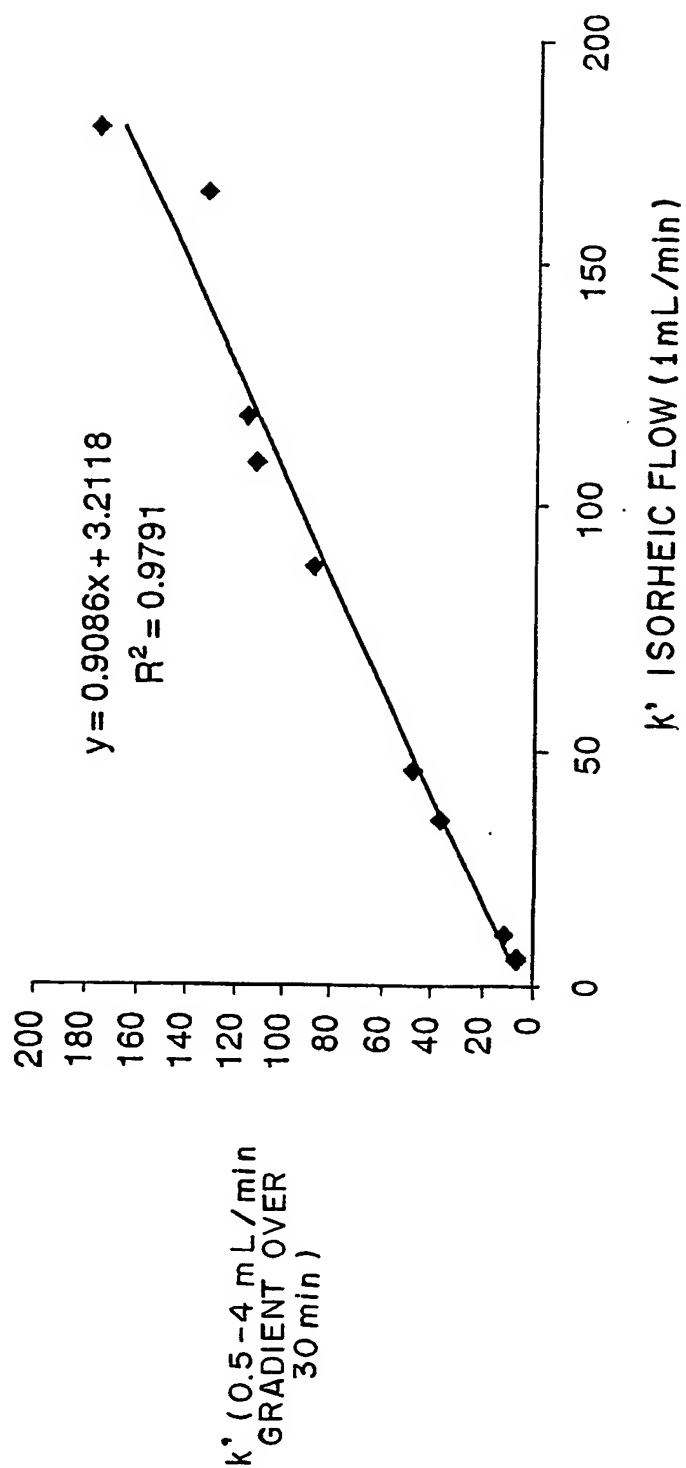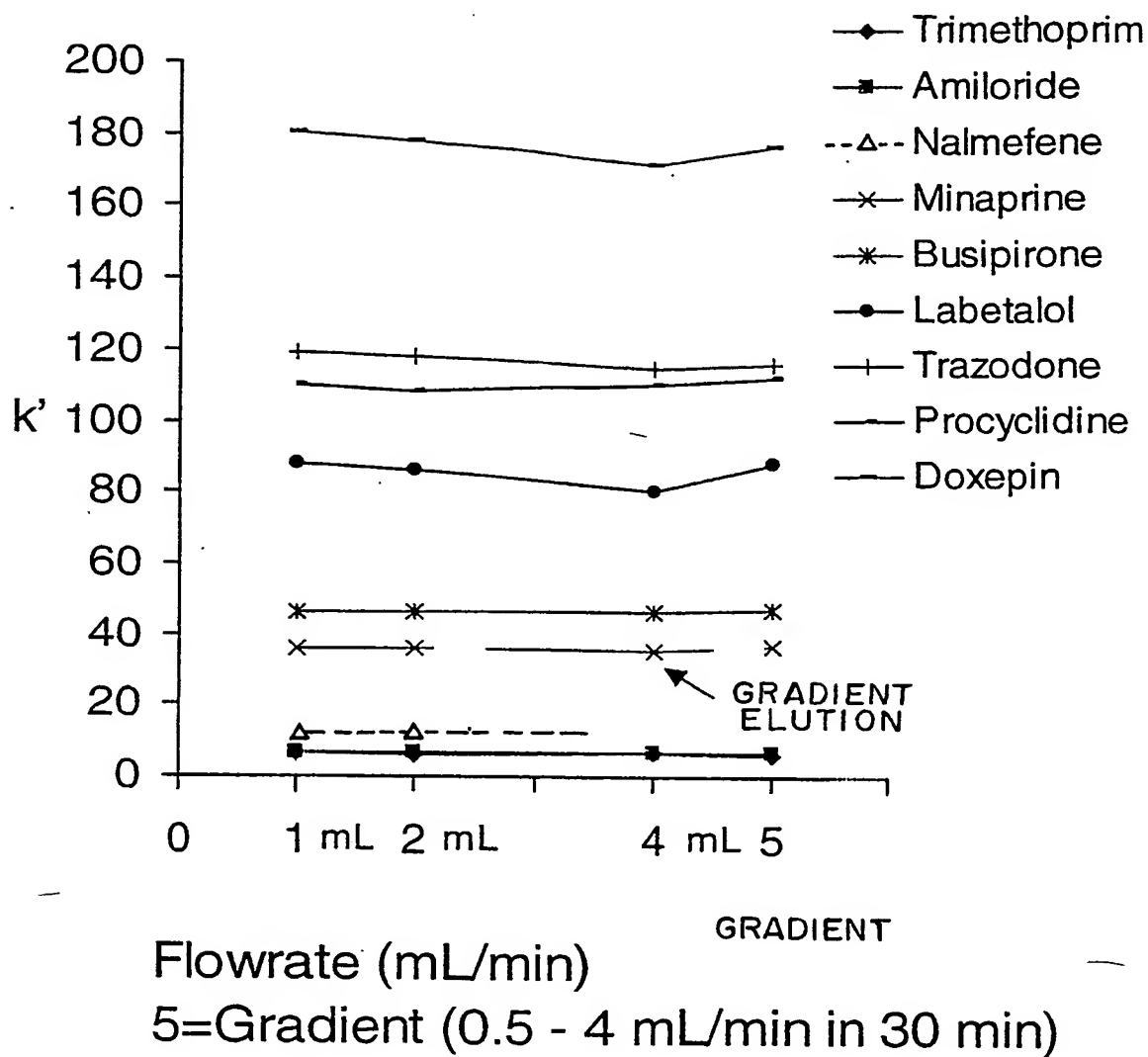
5=Gradient (0.5 - 4 mL/min in 30 min)

FIG. 19
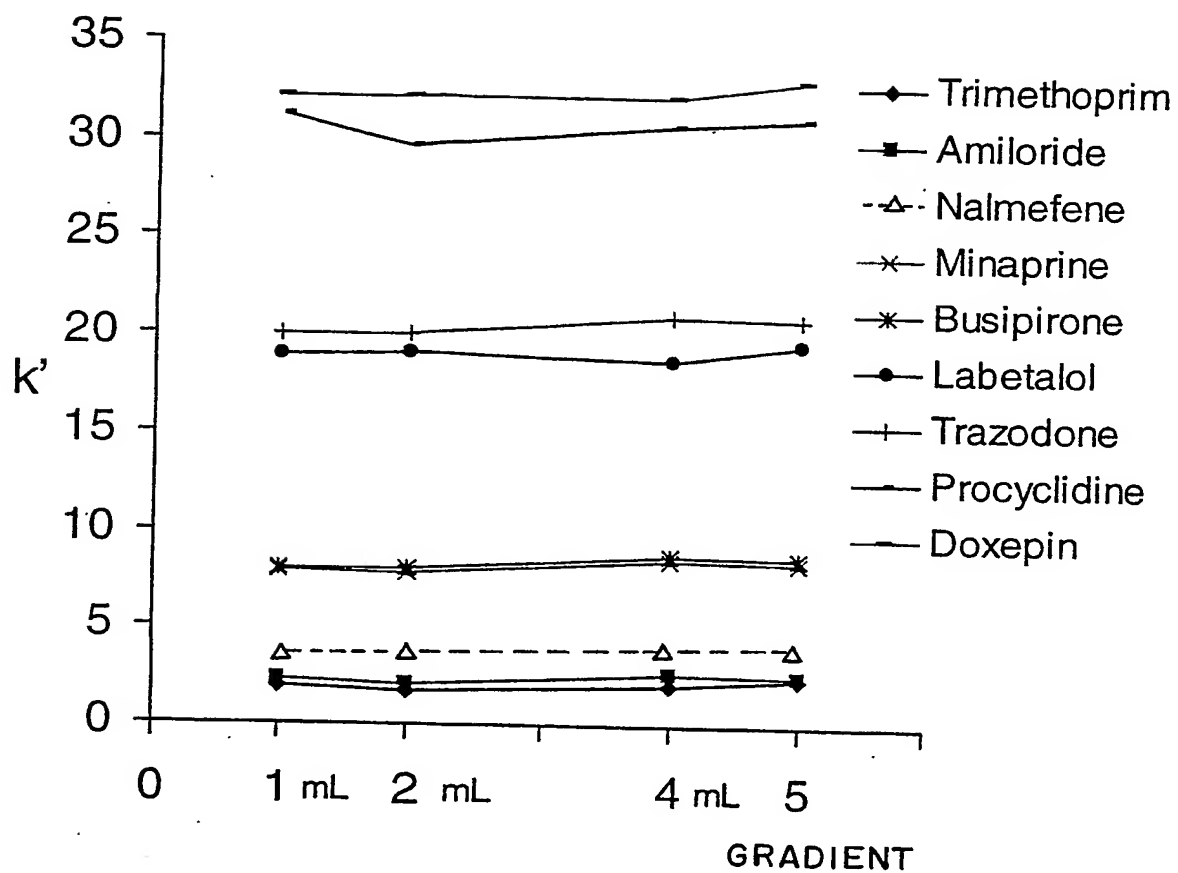
FIG. 20
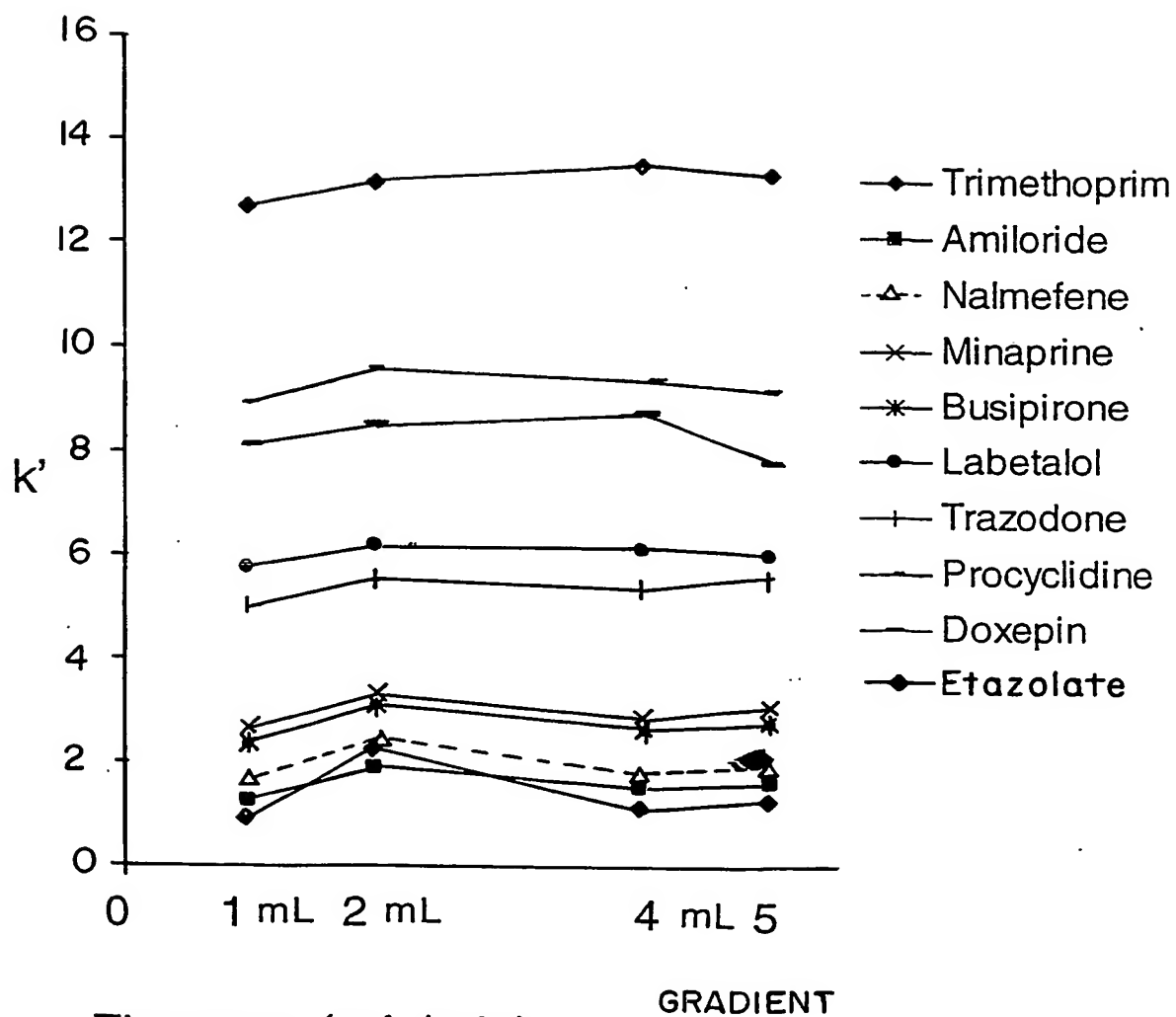
Flowrate (mL/min)
5=Gradient (0.5 - 4 mL/min in 30 min)

FIG 21
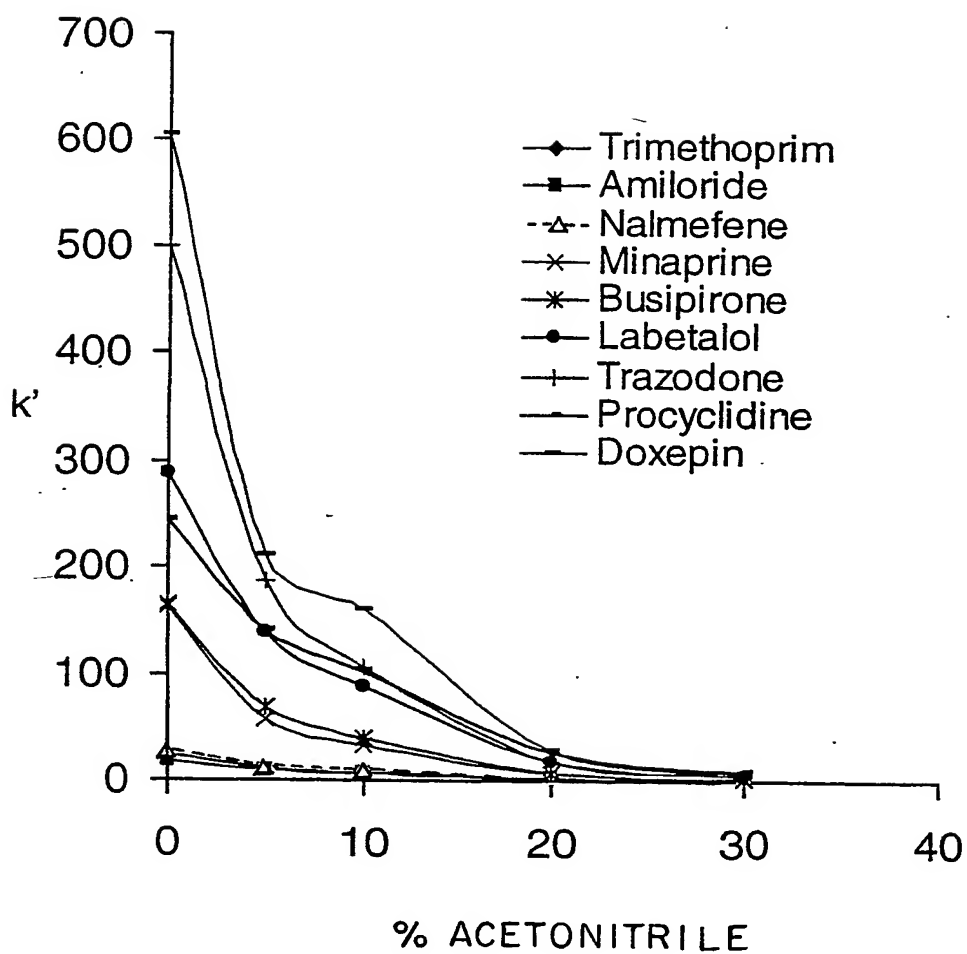
FLOW GRADIENT: 0.5-4 mL/min OVER 30 MINUTES,
THEN FLOW RATE MAINTAINED AT 4 mL/min)

FIG. 22
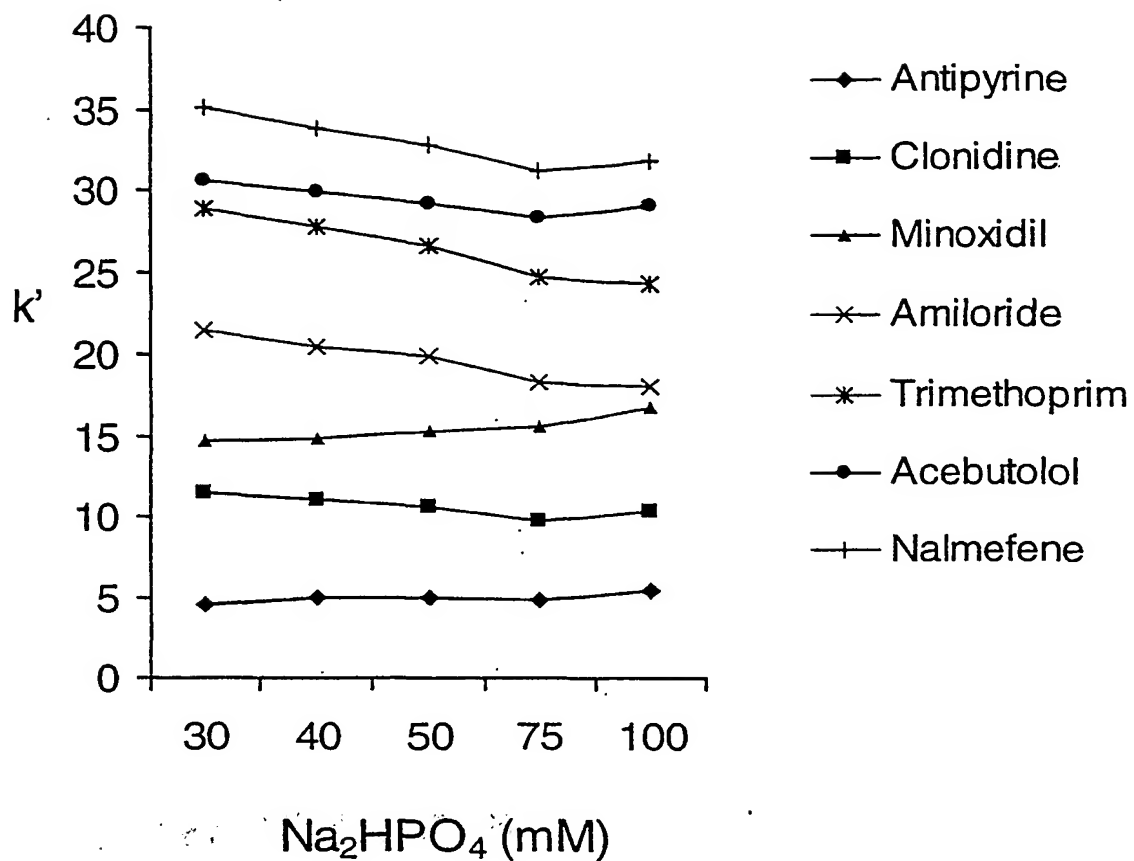
Mobile Phase Composition and pH:

	30	40	50	75	100
Na_2HPO_4 (mM)	30	40	50	75	100
NaCl (mM)	45	60	75	112.5	150
pH	7.19	7.16	7.11	7.05	7.01

FIG. 23

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(54) Title: HIGH THROUGHPUT CHROMATOGRAPHIC SYSTEMS

(57) Abstract: A method and apparatus is described for establishing a database comprising numerical values characteristic of the interaction of compounds and surfaces, such as stationary phases in a chromatographic system. In accordance with the invention a mobile phase flow gradient is used for enhancing efficiency and data precision in high throughput drug screening protocols. The chromatographic system includes an eluent flow splitter in fluid communication with the chromatographic unit and the detector to minimize variation in flow rate to the detector with variation of rate of flow of the mobile phase is in the chromatographic unit. With use of a detector capable of providing a signal of the presence, or more preferably an identifying characteristic, of compounds eluting from the system, the invention provides a powerful method for creating a database of numerical values characteristic of the interaction of solute test compounds with a mobile phase and a stationary phase in at least two chromatographic separation systems. Data can be generated efficiently and stored in algorithm accessible electronic storage systems (databases) for use alone or in combination with other molecular descriptors for prediction of biological activity.

INTERNATIONAL SEARCH REPORT

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7   G01N30/38   G01N30/86   G01N30/88   G01N30/46

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 7   G01N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

EPO-Internal

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
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[X] Further documents are listed in the continuation of box C.   [X] Patent family members are listed in annex.

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Date of the actual completion of the international search	Date of mailing of the international search report
23 October 2002	07/11/2002

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INTERNATIONAL SEARCH REPORT

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

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